



# Safety Assessment of Transgenic Organisms

OECD CONSENSUS DOCUMENTS

Volume 1 and 2



# **Safety Assessment of Transgenic Organisms**

OECD CONSENSUS DOCUMENTS

*Volume 1*



ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

## ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

The OECD is a unique forum where the governments of 30 democracies work together to address the economic, social and environmental challenges of globalisation. The OECD is also at the forefront of efforts to understand and to help governments respond to new developments and concerns, such as corporate governance, the information economy and the challenges of an ageing population. The Organisation provides a setting where governments can compare policy experiences, seek answers to common problems, identify good practice and work to co-ordinate domestic and international policies.

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*This book is published on the responsibility of the Working Group on Harmonisation of Regulatory Oversight in Biotechnology, which is a subsidiary group of the Chemicals Committee and Working Party on Chemicals, Pesticide and Biotechnology of the OECD.*

## FOREWORD

Genetically engineered crops (also known as transgenic crops) such as maize, soybean, rapeseed and cotton have been approved for commercial use in an increasing number of countries. During the period from 1996 to 2005, for example, there was more than fifty-fold increase in the area grown with transgenic crops worldwide, reaching 90 million hectares in 2005.<sup>1</sup> Such approvals usually follow a science-based risk/safety assessment.

The environmental safety/risks of transgenic organisms are normally based on the information on the characteristics of the host organism, the introduced traits, the environment into which the organism is introduced, the interaction between these, and the intended application. The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on identifying parts of this information, which could be commonly used in countries for environmental safety/risk assessment to encourage information sharing and prevent duplication of effort among countries. Biosafety Consensus Documents are one of the major outputs of its work.

Biosafety Consensus Documents are intended to be a "snapshot" of current information on a specific host organism or trait, for use during regulatory assessments. They are not intended to be a comprehensive source of information on everything that is known about a specific host or trait; but they do address the key or core set of issues that member countries believe are relevant to risk/safety assessment. This information is said to be mutually acceptable among member countries. To date, 25 Biosafety Consensus Documents have been published. They include documents which address the biology of crops, trees and micro-organisms as well as those which address specific traits which are used in transgenic crops.

This book is a compilation of those Biosafety Consensus Documents published before February 2006. It also includes two recently published texts: the first, entitled *An Introduction to the Biosafety Consensus Document of OECD's Working Group for Harmonisation in Biotechnology*, explains the purpose of the consensus documents and how they are relevant to risk/safety assessment. It also describes the process by which the documents are drafted using a "lead country" approach. The second text is a *Points to Consider for Consensus Documents on the Biology of Cultivated Plants*. This is a structured checklist of "points to consider" for authors when drafting or for those evaluating a consensus document. Amongst other things, this text describes how each point is relevant to risk/safety assessment.

This book offers ready access to those consensus documents which have been published thus far. As such, it should be of value to applicants for commercial uses of transgenic crops, regulators in national authorities as well as the wider scientific community. As each of the documents may be updated in the future as new knowledge becomes available, users of this book are encouraged to provide any information or opinions regarding the contents of this book or indeed, OECD's other harmonisation activities. If needed, a short pre-addressed questionnaire is attached at the end of this book that can be used to provide such comments.

The published Consensus Documents are also available individually from OECD's website (<http://www.oecd.org/biotrack>) at no cost.

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1. Clive James (2005), International Service for the Acquisition of Agri-biotech Applications (<http://www.isaaa.org/>)



## TABLE OF CONTENTS

(Please note that this table of contents is only for Volume 1 of this publication. A second volume is also available)

FOREWORD.....	3
INTRODUCTION TO THE BIOSAFETY CONSENSUS DOCUMENTS.....	9
PRESENTATION DES DOCUMENTS DE CONSENSUS SUR LA BIOTECHNOLOGIE ...	19
PART 1 CONSENSUS DOCUMENTS ON THE BIOLOGY OF CROPS .....	29
Section 1 Points to Consider for Consensus Documents on the Biology of Cultivated Plants	30
Section 2 Soybean ( <i>Glycine max</i> (L.) Marr.) .....	40
Section 3 Maize ( <i>Zea mays</i> subsp. <i>mays</i> ) .....	47
Section 4 Oilseed Rape ( <i>Brassica napus</i> L.) .....	79
Section 5 Rice ( <i>Oryza sativa</i> L.) .....	94
Section 6 Potato ( <i>Solanum tuberosum</i> subsp. <i>tuberosum</i> ) .....	126
Section 7 Bread Wheat ( <i>Triticum aestivum</i> ) .....	143
Section 8 Sugar Beet ( <i>Beta Vulgaris</i> L.) .....	174
Section 9 Sunflower ( <i>Helianthus annuus</i> L.) .....	197
Section 10 Papaya ( <i>Carica papaya</i> ) .....	227
Section 11 Oyster Mushroom ( <i>Pleurotus</i> spp.) .....	277
Section 12 <i>Capsicum Annuum</i> Complex .....	293
PART 2 CONSENSUS DOCUMENTS ON GENERAL INFORMATION OF TRAITS .....	323
Section 1 Virus Resistant through Coat Protein Gene-mediated Protection .....	324
Section 2 The Genes and Their Enzymes that Confer Tolerance to Glyphosate Herbicide...	351
Section 3 The Genes and Their Enzymes that Confer Tolerance to Phosphinothricin Herbicide .....	360
Section 4 Herbicide Biochemistry, Herbicide Metabolism and the Residues in (Phosphinothricin) - Tolerant Transgenic Plants.....	370

## Tables

Table 1.1	Consumption of maize <i>per capita</i> by country .....	48
Table 1.2	Classification of the genus <i>Zea</i> within the tribe <i>Maydeae</i> of the Western Hemisphere, and the genus <i>Tripsacum</i> .....	51
Table 1.3	Sexually obtained interspecific and intergeneric crosses with <i>B. Napus</i> .....	84
Table 1.4	Species belonging to the genus <i>Oryza</i> .....	95
Table 1.5	Comparison of main characters of domesticated cultivars of <i>O. sativa</i> and <i>O. glaberrima</i> .....	98
Table 1.6	Outcrossing rates estimated in wild and cultivated rice species by different methods .....	104
Table 1.7	Taxonomic position of <i>S. Tuberosum</i> subsp. <i>Tuberosum</i> .....	126
Table 1.8	Distinction between <i>S. tuberosum</i> subsp. <i>tuberosum</i> and subsp. <i>Andigena</i> .....	127
Table 1.9	Geographic distribution of the diploid einkorn lineage .....	147
Table 1.10	Geographic distribution of the tetraploid emmer lineage .....	147
Table 1.11	Geographic distribution of the hexaploid spelt lineage .....	148
Table 1.12	Manual intergeneric crossing with <i>Aegilops</i> (Ae.), <i>Secale</i> (S.), <i>Agropyron</i> (A.), <i>Haynaldia</i> (Ha.), <i>Hordeum</i> (H.) and <i>Elymus</i> (E.) .....	152
Table 1.13	Trigeneric hybrids from manual crossing <i>Triticum</i> (T.), <i>Aegilops</i> (Ae.), <i>Hordeum</i> (H.), <i>Agropyron</i> (A.), <i>Haynaldia</i> (Ha.) and <i>Secale</i> (S.) .....	153
Table 1.14	Classification of the <i>Beta</i> species .....	174
Table 1.15	Distribution and use of cultivated forms of <i>Beta vulgaris</i> ssp. <i>Vulgaris</i> .....	175
Table 1.16	Global distribution of the wild species of the genus <i>Beta</i> .....	175
Table 1.17	Distribution of the five <i>Beta</i> species present in Europe .....	181
Table 1.18	Classification of the genus <i>Helianthus</i> (Seiler and Rieseberg, 1997) .....	199
Table 1.19	Qualitative breakdown of plant dry matter (%) over the sunflower growth cycle .....	204
Table 1.20	Characteristics of the photosynthesis of selected crop plants .....	205
Table 1.21	Characteristics of the seed in terms of its location on the capitulum .....	206
Table 1.22	Common papaya varieties in commerce and breeding .....	234
Table 1.23	Formation of individual plant sexual types following papaya crosses .....	240
Table 1.24	Selected Papaya Genes for which Information is Available .....	247
Table 1.25	Classification of the genus <i>Pleurotus</i> and its geographical distribution .....	281
Table 1.26	Production of oyster mushrooms under commercial cultivation in some countries .....	282
Table 1.27	Environmental parameters for fruiting of oyster mushroom .....	284
Table 1.28	Estimated chromosome size of <i>Pleurotus</i> spp. ....	285
Table 1.29	Classification of <i>Capsicum annuum</i> .....	294
Table 1.30	The species of <i>Capsicum</i> and their known or apparently natural distributions. ..	296
Table 1.31	Morphological characters that generally differentiate the domesticated species of <i>Capsicum</i> .....	297
Table 1.32	Crossability of <i>Capsicum annuum</i> with other <i>Capsicum</i> in the three complexes of domesticated species .....	303
Table 2.1	Reported Viral Synergisms .....	340

**Figures**

Figure 1.1	Maize production worldwide .....	48
Figure 1.2	Evolutionary pathway of the two cultivated species of rice .....	96
Figure 1.3	Rice RFLP linkage map constructed with 1,383 DNA markers.....	100
Figure 1.4	An overview of the diploid einkorn lineage. ....	146
Figure 1.5	Macroscopic feature of <i>P. ostreatus</i> .....	278
Figure 1.6	Microscopic feature of <i>P. ostreatus</i> .....	279
Figure 1.7	Life cycle of the <i>Pleurotus ostreatus</i> .....	283
Figure 2.1	Glyphosate Structure .....	352
Figure 2.2	L-isomer of phosphinothricin (left) compared to glutamate (right) .....	362
Figure 2.3	Metabolism of Glufosinate-Ammonium in Non-Transgenic and in Transgenic, Tolerant Crop Plants (Corn, Oilseed rape, Tomato, Soybean, Sugar beet) .....	373



## **INTRODUCTION TO THE BIOSAFETY CONSENSUS DOCUMENTS**

## 1. About OECD's Working Group

OECD's Working Group comprises delegates from the 30 Member countries of OECD and the European Commission. Typically, delegates are from those government ministries and agencies, which have responsibility for the environmental risk/safety assessment of products of modern biotechnology. The Working Group also includes a number of observer delegations and invited experts who participate in its work. They include: Argentina; Russia; Slovenia; the United Nations Environment Programme (UNEP); the Secretariat of the Convention on Biological Diversity (SCBD); the United Nations Industrial Development Organisation (UNIDO); and the Business and Industry Advisory Committee to OECD (BIAC).

## 2. Regulatory Harmonisation

The Working Group was established in 1995<sup>2</sup> at a time when the first commercial transgenic crops were being considered for regulatory approval in a number of OECD Member countries. From the beginning, one of its primary goals was to promote international regulatory harmonisation in biotechnology among member countries. Regulatory harmonisation is the attempt to ensure that the information used in risk/safety assessments, as well as the methods used to collect such information, are as similar as possible. It could lead to countries recognising or even accepting information from one another's assessments. The benefits of harmonisation are clear. It increases mutual understanding among member countries, which avoids duplication, saves on scarce resources and increases the efficiency of the risk/safety assessment process. This in turn improves safety, while reducing unnecessary barriers to trade (OECD 2000). Many delegates have said that the process of working towards harmonisation, and the resulting discussions among member countries, is almost as important as the products produced.

## 3. The Need for Harmonisation Activities at OECD

The establishment of the Working Group and its programme of work followed a detailed analysis by member countries of whether there was a need to continue work on harmonisation in biotechnology at OECD, and if so, what that work should entail. This analysis was undertaken by the Ad Hoc Group for Environmental Aspects of Biotechnology (established by the Joint Meeting<sup>3</sup>), which was active, mainly during 1994.

The Ad Hoc Group took into consideration, and built upon, the earlier work at OECD, which began in the mid-1980s. Initially, these previous activities at OECD concentrated on the environmental and agricultural implications of field trials of transgenic organisms, but this was soon followed by a consideration of their large-scale use and commercialisation. (A summary of this extensive body of work is found in Annex I.)

## 4. Key Background Concepts and Principles

The Ad Hoc Group took into account (amongst other things) previous work on risk analysis that is summarised in *Safety Considerations for Biotechnology: Scale-up of Crop Plants* (OECD 1993a). The following quote gives the flavour: "*Risk/safety analysis is based on the characteristics of the organism, the*

2. The original title of the Working Group was the Expert Group for the Harmonisation of Regulatory Oversight in Biotechnology. It became an OECD Working Group in 1998.
3. The Joint Meeting was the supervisory body of the Ad Hoc Group and, as a result of its findings, established the Working Group as a subsidiary body. Today, its full title is the Joint Meeting of the Chemicals Committee and the Working Party on Chemical, Pesticides and Biotechnology.

*introduced trait, the environment into which the organism is introduced, the interaction between these, and the intended application.*" This body of work has formed the basis for environmental risk/safety assessment that is now globally accepted. So in considering the possibilities for harmonisation, the attention of the Ad Hoc Group was drawn to these characteristics and the information used by risk/safety assessors to address them.

This was reinforced by the concept of familiarity, which is also elaborated in the "Scale-up" document (OECD 1993a). This concept "...is based on the fact that most genetically engineered organisms are developed from organisms such as crop plants whose biology is well understood". "Familiarity allows the risk assessor to draw on previous knowledge and experience with the introduction of plants and micro-organisms into the environment..." For plants, familiarity takes account of a wide-range of attributes including, for example, knowledge and experience with "the crop plant, including its flowering/reproductive characteristics, ecological requirements, and past breeding experiences" (OECD 1993a – see also Annex I for a more detailed description). This illustrates the role of information related to the biology of the host organism as a part of an environmental risk/safety assessment.

The Ad Hoc Group also took into account the document "Traditional Crop Breeding Practices: An Historical Review to Serve as a Baseline for Assessing the Role of Modern Biotechnology" (OECD 1993b) which also focuses on host organisms. It presents information on 17 different crop plants, which are used (or are likely to be used) in modern biotechnology. It includes sections on phytosanitary considerations in the movement of germplasm and on current uses of these crop plants. There is also a detailed section on current breeding practices.

## **5. A Common Approach to Risk/Safety Assessment**

An important additional point for the Ad Hoc Group was to identify the extent to which member countries address the same questions and issues during risk/safety assessment. If there are big differences it would mean that attempts to work towards harmonisation would be difficult. On the other hand, a high level of similarity would suggest that harmonisation efforts would be more feasible.

This point was resolved by two studies, which the Ad Hoc Group was able to consider. The first covered crop plants (OECD 1995a, 1995b) while the second concerned micro-organisms (OECD 1995c, 1996). Both studies involved a survey targeted at those national authorities that are responsible for risk/safety assessment. The aim was to identify the questions which are addressed by them during the assessment process (as outlined in national laws/regulations/guidance documents) in order to establish the extent of similarity among national authorities. Both these studies used the information provided in OECD's "Blue Book" (OECD 1986) as a reference point, in particular, the sections of the book (appendices b, c and d) which cover: i) General Scientific Considerations; ii) Human Health Considerations; and iii) Environmental and Agricultural Considerations. Both studies identified a remarkably high degree of similarity among member countries in the questions/issues addressed in risk/safety assessment.

## **6. The Emergence of the Concept of Consensus Documents**

So the Working Group was established in the knowledge that national authorities have much in common, in terms of the questions/issues addressed, when undertaking risk/safety assessment. It also took into account those characteristics identified as part of risk/safety assessment (*i.e. the organism, the introduced trait and the environment*) around which harmonisation activities could focus.

It was further recognised that much of the information used in risk/safety assessment that relates to the biology of organisms (both crop plants and micro-organisms) would be similar or virtually the same in

all assessments involving the same organism. In other words, the questions addressed during risk/safety assessment which relate to the biology of the host organism - for example, the potential for gene transfer within the crop plant species, and among related species, as well as the potential for weediness – remain the same for each application involving the same host species. This also applies to some extent to information related to introduced traits.

Consequently, the Working Group evolved the idea of compiling information common to the risk/safety assessment of a number of transgenic products, and decided to focus on two specific categories: the biology of the host species or crop; and traits used in genetic modifications. The aim of this compilation was to encourage information sharing and prevent duplication of effort among countries by avoiding the need to address the same common issues in each application involving the same organism or trait. It was recognized that biology and trait consensus documents could be agreed upon quickly by the member countries (within one or two years). This compilation process was quickly formalised in the drafting of Consensus Documents.

## **7. The Purpose of Consensus Documents**

The Consensus Documents are not intended to be a substitute for a risk/safety assessment, because they address only a part of the necessary information. Nevertheless, they should make an important contribution to environmental risk/safety assessment.

As originally stated by the Working Group, Consensus Documents are intended to be a “snapshot” of current information, for use during the regulatory assessment of products of biotechnology. They are not intended to be a comprehensive source of information on everything that is known about a specific host organism or trait; but address – on a consensus basis – the key or core set of issues that member countries believe are relevant to risk/safety assessment.

The aim of the documents is to share information on these key components of an environmental safety review in order to prevent duplication of effort among countries. The documents were envisaged as being used: a) by applicants as information in applications to regulatory authorities; b) by regulators as a general guide and reference source in their reviews; and c) by governments for information sharing, research reference and public information.

Originally, it was said that the information in the Consensus Documents is intended to be *mutually recognised or mutually acceptable* among OECD Member countries, though the precise meaning of these terms, in practice, is still open for discussion. During the period of the Ad Hoc Group and the early days of the Working Group (1993-1995), the phrase *Mutual Acceptance of Data* was discussed. This is a concept borrowed from OECD’s Chemicals Programme which involves a system of OECD Council Decisions that have legally binding implications for member countries. In the case of the Consensus Documents there has never been any legally binding commitment to use the information in the documents, though from time to time, the Working Group has discussed whether and how to increase the level of commitment member countries are willing to make in using the information in the documents. Participation in the development of documents, and the intention by member countries to use the information, is done in “good faith.” It is expected, therefore, that reference will be made to relevant consensus documents during risk/safety assessments.

## **8. The Process through which Consensus Documents are Initiated and Brought to Publication**

There are a number of steps in the drafting of a specific consensus documents. The first step occurs when a delegation, in a formal meeting of the Working Group, makes a proposal to draft a document on a new topic, typically a crop species or a trait. If the Working Group agrees to the proposal, a provisional

draft is prepared by either a single country or two or more countries working together. This is often called the "lead country approach". Typically, the lead country(ies) has had experience with the crop or trait which is the subject of the new document and is able to draw on experts to prepare a provisional draft.

The provisional draft is first reviewed by the Bureau of the Working Group<sup>4</sup> to ensure that the document addresses range of issues normally covered by Consensus Documents and is of sufficiently high quality to merit consideration by the Working Group as a whole.

Based on the comments of the Bureau, a first draft is then prepared for consideration by the full Working Group. This is the opportunity for each delegation to review the text and provide comments based on their national experiences. The incorporation of these comments leads to a second draft, which is again circulated for review and comment to the Working Group. At this point, the Working Group may be asked to recommend that the document be declassified. Such a recommendation is only forthcoming when all delegations have come to a consensus that the document is complete and ready for publication. Sometimes, however, the text may need a third or even a fourth discussion in the Working Group before a recommendation for declassification is possible.

When the Working Group has agreed that a document can be recommended for declassification, it is forwarded to the supervisory Committee, the Joint Meeting, which is invited to declassify the document. Following the agreement of the Joint Meeting, the document is then published.

It is important to note that the review of Consensus Documents is not limited to formal meetings of the Working Group. Much discussion also occurs through electronic means, especially via the Working Group's Electronic Discussion Group (EDG). This enables a range of experts to have input into drafts.

For a number of documents, it has also been important to include information from non-member countries. This has been particularly true in the case of crop plants where the centre of origin and diversity occurs in a non-member country(ies). In these cases, UNEP and UNIDO have assisted in the preparation of documents by identifying experts from countries which include the centres of origin and diversity. For example, this occurred with the Consensus Document on the Biology of Rice.

## **9. Current and Future Trends in the Working Group**

The Working Group continues its work, not only on the preparation of specific Consensus Documents, but also on the efficiency of the process by which they are developed. At the present time, an increasingly large number of crops and other host species are being modified, for increasing number of traits.

At the OECD Workshop on Consensus Documents and Future Work in Harmonisation, which was held in Washington DC, 21-23 October 2003, the Working Group was able to consider, amongst other things, how to set priorities for drafting future Consensus Documents among the large number of possibilities. The Working Group is currently considering how best to set priorities in the future.

The Workshop also recognised that published Consensus Documents may be in need of review and updating from time to time, to ensure that they include the most recent information. The Working Group is currently considering how best to organise this in the future.

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4. The Bureau comprises the Chair and vice-Chairs of the Working Group. The Bureau is elected by the Working Group once per year. At the time of writing, the Chair is from Austria and the vice-Chairs are from Canada, Japan the Netherlands and the United States.

For the future drafting of new and updated documents, the Workshop identified the usefulness of developing a standardised structure of Consensus Documents, which is called “Points to Consider”. The Working Group is expected to develop, firstly, a Points to Consider document for the biology Consensus Documents and then that of the trait Consensus Documents.

The Workshop also recognised the importance strengthening the input of non-member countries into the future development of Consensus Documents. Once again, the Working Group is considering how best to implement this recommendation.

## APPENDIX I

### OECD Biosafety Principles and Concepts Developed Prior to the Working Group 1986-1994

Since the mid-1980s the OECD has been developing harmonised approaches to the risk/safety assessment of products of modern biotechnology. Prior to the establishment of the Working Group, OECD published a number of reports on safety considerations, concepts and principles for risk/safety assessment as well as information on field releases of transgenic crops, and a consideration of traditional crop breeding practices. This Annex notes some of the highlights of these achievements that were background considerations in the establishment of the Working Group and its development of Consensus Documents.

#### *Underlying scientific principles*

In 1986, OECD published its first safety considerations for genetically engineered organisms (OECD 1986). These included the issues (relevant to human health, the environment and agriculture) that might be considered in a risk/safety assessment. In its recommendations for agricultural and environmental applications, it suggested that risk/safety assessors:

- “Use the considerable data on the environmental and human health effects of living organisms to guide risk assessments.
- Ensure that recombinant DNA organisms are evaluated for potential risk, prior to application in agriculture and the environment by means of an independent review of potential risks on a case-by-case basis.
- Conduct the development of recombinant DNA organisms for agricultural and environmental applications in a stepwise fashion, moving, where appropriate, from the laboratory to the growth chamber and greenhouse, to limited field testing and finally to large-scale field testing.
- Encourage further research to improve the prediction, evaluation, and monitoring of the outcome of applications of recombinant DNA organisms.”

#### *The role of confinement in small scale testing*

In 1992, OECD published its Good Developmental Principles (GDP) (OECD 1992) for the design of small-scale field research involving GM plants and GM micro-organisms. This document, amongst other things, describes the use of *confinement* in field tests. Confinement includes measures, to avoid the dissemination or establishment of organisms from a field trial, for example, the use of physical, temporal, or biological isolation (such as the use of sterility).

#### *Scale-up of crop-plants – “risk/safety analysis”*

By 1993, the focus of attention had switched to the *scale-up* of crop plants as plant breeders began to move to larger-scale production and commercialisation of GM plants. OECD published general principles for, *scale-up* (OECD 1993a), which re-affirmed that, “*safety in biotechnology is achieved by the appropriate application of risk/safety analysis and risk management. Risk/safety analysis comprises hazard identification and, if a hazard has been identified, risk assessment. Risk/safety analysis is based on*

*the characteristics of the organism, the introduced trait, the environment into which the organism is introduced, the interaction between these, and the intended application. Risk/safety analysis is conducted prior to an intended action and is typically a routine component of research, development and testing of new organisms, whether performed in a laboratory or a field setting. Risk/safety analysis is a scientific procedure which does not imply or exclude regulatory oversight or imply that every case will necessarily be reviewed by a national or other authority” (OECD 1993a).*

### ***The role of familiarity in risk/safety assessment***

The issue of *scale-up* also led to an important concept, *familiarity*, which is one key approach that has been used subsequently to address the environmental safety of transgenic plants.

The concept of familiarity is based on the fact that most genetically engineered organisms are developed from organisms such as crop plants whose biology is well understood. It is not a risk/safety assessment in itself (U.S. NAS 1989). However, the concept facilitates risk/safety assessments, because to be familiar, means having enough information to be able to make a judgement of safety or risk (U.S. NAS 1989). Familiarity can also be used to indicate appropriate management practices including whether standard agricultural practices are adequate or whether other management practices are needed to manage the risk (OECD 1993a). Familiarity allows the risk assessor to draw on previous knowledge and experience with the introduction of plants and micro-organisms into the environment and this indicates appropriate management practices. As familiarity depends also on the knowledge about the environment and its interaction with introduced organisms, the risk/safety assessment in one country may not be applicable in another country. However, as field tests are performed, information will accumulate about the organisms involved, and their interactions with a number of environments.

Familiarity comes from the knowledge and experience available for conducting a risk/safety analysis prior to scale-up of any new plant line or crop cultivar in a particular environment. For plants, for example, familiarity takes account of, but need not be restricted to, knowledge and experience with:

- “The crop plant, including its flowering/reproductive characteristics, ecological requirements, and past breeding experiences.
- The agricultural and surrounding environment of the trial site.
- Specific trait(s) transferred to the plant line(s).
- Results from previous basic research including greenhouse/glasshouse and small-scale field research with the new plant line or with other plant lines having the same trait.
- The scale-up of lines of the plant crop varieties developed by more traditional techniques of plant breeding.
- The scale-up of other plant lines developed by the same technique.
- The presence of related (and sexually compatible) plants in the surrounding natural environment, and knowledge of the potential for gene transfer between crop plant and the relative.
- Interactions between/among the crop plant, environment and trait.” (OECD, 1993a).

***Risk/safety assessment and risk management***

Risk/safety assessment involves the identification of potential environmental adverse effects or hazards, and determining, when a hazard is identified, the probability of it occurring. If a potential hazard or adverse effect is identified, measures may be taken to minimise or mitigate it. This is risk management. Absolute certainty or zero risk in a safety assessment is not achievable, so uncertainty is an inescapable aspect of all risk assessment and risk management (OECD 1993a). For example, there is uncertainty in extrapolating the results of testing in one species to identify potential effects in another. Risk assessors and risk managers thus spend considerable effort to address uncertainty. Many of the activities in intergovernmental organisations, such as the OECD, address ways to handle uncertainty (OECD 2000).

## APPENDIX II

### References Cited in Chronological Order

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## **PRESENTATION DES DOCUMENTS DE CONSENSUS SUR LA BIOTECHNOLOGIE**

## A propos du Sous-groupe de l'OCDE

Le Sous-groupe comprend des délégués des 30 pays Membres de l'OCDE et de la Commission européenne. En général, les délégués sont des fonctionnaires des ministères et organismes gouvernementaux chargés de l'évaluation des risques pour l'environnement et de la sécurité des produits issus de la biotechnologie moderne. Le Sous-groupe comprend aussi plusieurs délégations et experts invités qui participent à ses travaux en qualité d'observateurs notamment l'Argentine, la Russie, la Slovaquie, le Programme des Nations Unies pour l'environnement (PNUE), le Secrétariat de la Convention sur la diversité biologique (SCDB), l'Organisation des Nations Unies pour le développement industriel (ONUDI) et le Comité consultatif économique et industriel auprès de l'OCDE (BIAC).

## Harmonisation de la réglementation

Le Sous-groupe a été créé en 1995<sup>5</sup> au moment des premières demandes d'autorisation réglementaires de cultures commerciales transgéniques dans plusieurs pays Membres de l'OCDE. Dès le début, l'un de ses premiers objectifs a été de promouvoir l'harmonisation internationale de la réglementation entre les pays Membres. L'harmonisation réglementaire vise à assurer que les données utilisées pour l'évaluation des risques et de la sécurité, de même que les méthodes utilisées pour les collecter sont aussi uniformes que possible entre les pays. Elle peut permettre aux pays de reconnaître ou même d'accepter les informations des évaluations réalisées par d'autres pays. Les avantages de l'harmonisation sont évidents. Elle accroît la compréhension mutuelle entre les pays Membres, et permet donc d'éviter les recoupements, d'économiser les ressources rares et d'accroître l'efficacité des procédures d'évaluation des risques et de la sécurité. Cela permet d'améliorer la sécurité, tout en éliminant les obstacles inutiles au commerce (OCDE 2000). De l'avis de nombreux délégués la recherche d'une harmonisation, et les débats qui en résultent entre les pays Membres, sont presque aussi importants que les produits obtenus.

## Pourquoi mener des activités d'harmonisation à l'OCDE

Le Sous-groupe a été créé et son programme, établi après que les pays Membres aient mené une réflexion approfondie pour déterminer s'il fallait poursuivre ou non les travaux sur l'harmonisation en biotechnologie dans le cadre de l'OCDE, et dans l'affirmative, ce que ces travaux impliqueraient. Cette réflexion a été menée par le Groupe ad hoc sur les aspects environnementaux de la biotechnologie (créé par la Réunion conjointe<sup>6</sup>), qui a mené un grand nombre d'activités principalement pendant l'année 1994.

Le Groupe ad hoc a pris en considération et mis à profit les précédents travaux faits à l'OCDE, à partir du milieu des années 80. Les activités antérieures de l'OCDE se sont initialement concentrées sur les conséquences pour l'environnement et l'agriculture des essais au champ d'organismes transgéniques, mais ont ensuite très vite porté sur leur utilisation à grande échelle et leur commercialisation. (On trouvera un résumé de tous ces travaux à l'annexe I.)

## Principaux concepts et principes de base

Le Groupe ad hoc a pris en compte (entre autres éléments) les précédents travaux sur l'analyse des risques dont on trouve un résumé dans le document *Considérations de sécurité relatives à la*

5. Le Sous-groupe s'appelait à l'origine Groupe d'experts pour l'harmonisation de la surveillance réglementaire en biotechnologie. Il est devenu un Sous-groupe de l'OCDE en 1998.
6. La Réunion conjointe était l'organe de tutelle du Groupe ad hoc et a fait du Sous-groupe, en raison de ses résultats, un organe subsidiaire. Aujourd'hui, son nom officiel est Réunion conjointe du Comité des produits chimiques et du Groupe de travail sur les produits chimiques, les pesticides et la biotechnologie.

*biotechnologie : passage à l'échelle supérieure des plantes cultivées* (OCDE 1993a). L'extrait suivant en donne un aperçu : « l'analyse de risque/de sécurité s'appuie sur les caractéristiques de l'organisme, le caractère introduit, l'environnement dans lequel l'organisme est libéré, les interactions de ces facteurs entre eux et l'utilisation prévue. » Ces travaux ont servi de point de départ à l'évaluation environnementale des risques et de la sécurité, aujourd'hui acceptée mondialement. Par conséquent en examinant les possibilités d'harmonisation le Groupe ad hoc s'est intéressé à ces caractères et aux informations utilisées par les évaluateurs des risques et de la sécurité pour les examiner.

A cela s'ajoute le concept de familiarité, qui est aussi décrit dans le document « Mise à l'échelle » (OCDE 1993a). Ce concept « est basé sur le fait que la plupart des organismes transformés génétiquement sont développés à partir d'organismes comme les végétaux cultivés, dont la biologie est bien comprise ». « La familiarité permet à l'évaluateur de risques d'appliquer ses connaissances et son expérience de l'introduction des végétaux et des microorganismes dans l'environnement. » S'agissant des végétaux, la familiarité tient compte d'un grand nombre d'éléments, par exemple, des connaissances et de l'expérience concernant « les végétaux cultivés, y compris leurs caractéristiques de floraison et de reproduction, leurs exigences écologiques et les expériences passées en matière de sélection » (OCDE 1993a – une description plus détaillée est proposée dans l'annexe I). Cela montre bien le rôle des informations concernant la biologie de l'organisme hôte lors de l'évaluation des risques pour l'environnement et de la sécurité.

Le Groupe ad hoc a également pris en compte le document intitulé « Méthodes traditionnelles de sélection des plantes : un aperçu historique destiné à servir de référence pour l'évaluation du rôle de la biotechnologie moderne » (OCDE 1993b) qui met aussi l'accent sur les organismes hôtes. On y trouve des informations sur 17 plantes cultivées qui sont (ou pourraient être) utilisées en biotechnologie moderne. Ce document comprend des sections consacrées aux aspects phytosanitaires du transfert de matériel génétique et aux utilisations actuelles de ces plantes. Une section détaillée est également consacrée aux pratiques de sélection actuelles.

### **Une approche commune de l'évaluation des risques et de la sécurité**

L'une des missions importantes du Groupe ad hoc était aussi d'évaluer dans quelle mesure les pays Membres étudient les mêmes questions et problèmes lors de l'évaluation des risques et de la sécurité. En cas de différences importantes la recherche d'une harmonisation aurait pu se révéler difficile ; à l'inverse, en présence de nombreuses similitudes, le travail d'harmonisation serait plus aisé.

Deux études ont permis au Groupe ad hoc de répondre à cette question. La première concernait les plantes cultivées (OCDE 1995a, 1995b) et la seconde les micro-organismes (OCDE 1995c, 1996). Ces deux études étaient basées sur une enquête adressée aux autorités nationales chargées de l'évaluation des risques et de la sécurité. L'objectif était de faire ressortir les questions couvertes par le processus d'évaluation (d'après la législation, la réglementation ou les documents d'orientation nationaux) pour établir le degré de similitude entre les autorités nationales. Ces deux études ont pris pour référence les informations figurant dans le « Livre bleu » de l'OCDE (OCDE 1986), en particulier dans les annexes b, c et d) intitulées : i) Considérations scientifiques générales ; ii) Considérations relatives à la santé humaine ; et iii) Considérations relatives à l'environnement et l'agriculture. Ces deux études ont permis de constater que les questions et problèmes traités par les pays Membres pour évaluer les risques et la sécurité présentaient un remarquable degré de similitude.

### **Apparition du concept de document de consensus**

Le Sous-groupe a pu ainsi établir que les questions et problèmes traités par les autorités nationales aux fins de l'évaluation des risques et de la sécurité présentaient de très nombreux points communs. Il a également pris en compte les caractères identifiés dans le cadre de l'évaluation des risques et de la sécurité

(l'organisme, le caractère introduit et l'environnement) sur lesquelles pourraient se concentrer les activités d'harmonisation.

Il a ensuite été constaté qu'une grande partie des informations concernant la biologie des organismes (aussi bien des plantes que des micro-organismes) utilisées dans l'évaluation des risques et de la sécurité étaient les mêmes ou pratiquement les mêmes dans toutes les évaluations portant sur le même organisme. En d'autres termes, les questions concernant la biologie de l'organisme hôte (par exemple, le potentiel de transfert de gènes à l'intérieur d'une espèce de plante cultivée, et entre des espèces apparentées, de même que le caractère adventice potentiel) examinées dans le cadre de l'évaluation des risques et de la sécurité étaient les mêmes pour chaque demande impliquant les mêmes espèces hôtes. Il en allait de même, jusqu'à un certain point, pour les informations relatives aux caractères introduits.

En conséquence, le Sous-groupe a eu l'idée de regrouper les informations communes utilisées dans l'évaluation des risques et de la sécurité d'un certain nombre de produits transgéniques et décidé de se concentrer sur deux catégories particulières : la biologie des espèces ou plantes hôtes ; et les caractères utilisés dans les modifications génétiques. L'objectif était d'encourager le partage de l'information et d'éviter les doublons en permettant aux pays de ne pas traiter les mêmes questions communes pour chaque demande concernant le même organisme ou le même caractère. Il a été décidé que des documents de consensus sur la biologie ou les caractères pouvaient être rapidement adoptés par les pays Membres (en un ou deux ans). Ce processus de compilation a rapidement débouché sur la rédaction de documents de consensus.

### **Objet des documents de consensus**

Les documents de consensus ne prétendent pas se substituer à l'évaluation des risques et de la sécurité, car ils ne concernent qu'une partie de l'information nécessaire. Cependant, ils devraient faciliter grandement l'évaluation environnementale des risques et de la sécurité.

Comme l'a indiqué initialement le Sous-groupe, les documents de consensus visent à fournir un aperçu des données courantes pouvant être utilisées dans le processus d'évaluation réglementaire des produits issus de la biotechnologie. Ils ne prétendent pas offrir une source d'informations exhaustive sur l'ensemble des connaissances concernant un organisme hôte ou un caractère particulier ; ils abordent plutôt le « noyau dur » des questions jugées pertinentes, sur la base d'un consensus, par les pays Membres pour l'évaluation des risques et de la sécurité.

Ces documents visent à faciliter l'échange d'informations sur ces composantes clés des évaluations de la sécurité environnementale afin d'éviter que les activités menées dans les pays ne fassent double emploi. Ils étaient en principe destinés : a) aux pétitionnaires à titre d'informations pour les demandes adressées aux autorités de réglementation ; b) aux autorités chargées de la réglementation comme guide général et source de référence pour leurs examens ; et c) aux gouvernements aux fins de l'échange d'information, comme références de recherche et pour l'information du public.

Il a été décidé initialement que les informations contenues dans les documents de consensus devaient être *mutuellement reconnues ou mutuellement acceptées* par les pays Membres de l'OCDE, bien que le sens de ces expressions reste encore à préciser. L'expression *acceptation mutuelle des données* a été étudiée pendant la période de mandat du Groupe ad hoc et durant les premières années du Sous-groupe (1993-1995). Il s'agit en fait d'une notion empruntée au Programme des produits chimiques de l'OCDE pour désigner un système de Décisions du Conseil de l'OCDE qui ont un caractère contraignant pour les pays Membres. Dans le cas des documents de consensus, il n'a jamais été obligatoire d'utiliser les informations y figurant, même si le Sous-groupe s'interroge de temps à autres sur l'opportunité et la façon d'impliquer davantage les pays Membres qui utilisent les informations fournies dans ces documents. La

participation des pays Membres à l'élaboration des documents et leur intention d'utiliser les informations qu'ils contiennent sont présumées de bonne foi. On peut donc penser que les documents de consensus applicables serviront de référence dans les évaluations des risques et de la sécurité.

### **Processus d'établissement des documents de consensus débouchant sur leur publication**

La rédaction d'un document de consensus se fait en plusieurs étapes. Tout commence lorsqu'une délégation, à l'occasion d'une réunion officielle du Sous-groupe, propose d'établir un document sur un nouveau sujet, en général une espèce végétale ou un caractère. Si le Sous-groupe approuve la proposition, un premier projet est préparé par un, deux ou plusieurs pays en collaboration. Cette étape repose sur le principe de « pilotage ». En général, le ou les pays pilote(s) possèdent une expérience de la plante ou du caractère visés par le nouveau document et peuvent faire appel à des experts pour préparer une première version.

Cette version préliminaire est d'abord examinée par le Bureau du Sous-groupe<sup>7</sup> qui vérifie que le document étudie bien tous les aspects habituellement pris en compte par les documents de consensus et que sa qualité est suffisamment bonne pour le présenter à l'ensemble du Sous-groupe.

Un premier projet est établi à la lumière des commentaires du Bureau puis présenté à l'ensemble du Sous-groupe. De cette façon, chaque délégation peut étudier le texte et formuler des commentaires en fonction de l'expérience de son pays. Après incorporation de ces commentaires on obtient la deuxième mouture, qui est à nouveau diffusée pour examen et commentaires au Sous-groupe. A ce stade, le Sous-groupe peut être invité à recommander la déclassification du document. Cette demande intervient uniquement lorsque toutes les délégations ont décidé d'un commun accord (consensus) que le document était complet et prêt pour publication. Il arrive cependant que le texte nécessite un troisième, voire un quatrième examen au sein du Sous-groupe avant que sa déclassification puisse être recommandée.

Lorsque le Sous-groupe est convenu que le document pouvait être recommandé pour déclassification, le document est transmis à l'organe de tutelle, la Réunion conjointe, qui est invitée à le déclassifier. Une fois approuvé par la Réunion conjointe, le document est publié.

Il importe de noter que l'examen des documents de consensus dépasse le cadre des réunions officielles du Sous-groupe. De nombreux échanges de vues se font aussi par voie électronique, notamment dans le cadre du groupe de discussion électronique du Sous-groupe. Cette formule permet à divers experts de compléter les projets.

Il s'est révélé également important, dans le cas de plusieurs documents, d'inclure des informations de pays non membres. Cela s'est notamment produit pour les plantes cultivées dont les centres d'origine et de diversité se trouvent dans un ou des pays non membre(s). Dans ces cas, le PNUE et l'ONUDI ont contribué à l'établissement des documents en indiquant quels experts contacter dans les pays renfermant les centres d'origine et de diversité. Une telle situation s'est produite par exemple, lors de l'établissement du document de consensus sur la biologie du riz.

### **Évolutions actuelles et futures au sein du Sous-groupe**

Le Sous-groupe poursuit ses travaux, non seulement de préparation des documents de consensus, mais aussi d'étude de l'efficacité du processus d'établissement de ces documents. A l'heure actuelle, un nombre

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7. Le Bureau comprend le Président et les Vice-présidents du Sous-groupe. Le Bureau est élu par le Sous-groupe une fois par an. Au moment où ce document est écrit, le Président est autrichien et les Vice-présidents sont des représentants du Canada, des États-Unis, du Japon et des Pays-Bas.

croissant de plantes cultivées et d'autres espèces sont modifiées par le transfert de caractères transférés de plus en plus nombreux.

Lors de l'atelier de l'OCDE consacré aux documents de consensus et aux travaux futurs sur l'harmonisation, qui s'est tenu à Washington DC, du 21 au 23 octobre 2003, le Sous-groupe a réfléchi, entre autres choses, à la façon d'établir un ordre de priorité pour la préparation des documents de consensus. Il examine actuellement comment améliorer à l'avenir le classement des priorités.

Les participants à l'atelier ont également reconnu qu'il pourrait être nécessaire de revoir ou de mettre à jour périodiquement les documents de consensus, pour veiller à ce qu'ils contiennent les informations les plus récentes. Le Sous-groupe examine actuellement la meilleure façon de procéder pour concrétiser ce projet.

Les participants ont constaté qu'il serait utile d'établir une structure type de document de consensus, sous forme de liste de « points à examiner » pour faciliter l'établissement des prochains documents et leur mise à jour. Le Sous-groupe devrait préparer un document recensant les points à examiner tout d'abord pour les documents de consensus relatifs à la biologie puis pour ceux concernant les caractères.

L'atelier a par ailleurs indiqué qu'il était important d'associer davantage les pays non membres à l'établissement des prochains documents de consensus. Ici encore, le Sous-groupe réfléchit à la meilleure façon de mettre en oeuvre cette recommandation.

## ANNEXE I

### Principes et concepts relatifs à la biosécurité établis par l'OCDE avant la création du Sous-groupe 1986-1994

Depuis le milieu des années 1980, l'OCDE a développé des approches harmonisées pour l'évaluation des risques et de la sécurité des produits de biotechnologie moderne. Avant la création du Sous-groupe, l'OCDE a publié plusieurs rapports d'experts portant sur des questions de sécurité, les concepts et principes relatifs à l'évaluation des risques et de la sécurité de même que sur la dissémination des cultures transgéniques dans les champs et sur la question des pratiques de croisement des cultures traditionnelles. La présente annexe récapitule les éléments essentiels de ces travaux qui ont servi de point de départ à la création du Sous-groupe et aux documents de consensus établis.

#### *Principes scientifiques sous-jacents*

En 1986, l'OCDE a publié ses premières études sur la sécurité des organismes transformés génétiquement (OCDE, 1986). Ceux-ci comprenaient des questions (intéressant la santé humaine, l'environnement et l'agriculture) qui pourraient être prises en compte dans l'évaluation des risques et de la sécurité. Dans ses recommandations pour les applications agricoles et environnementales, il est suggéré que les évaluateurs des risques et de la sécurité :

- « Utilisent des données nombreuses sur les effets au niveau de l'environnement et de la santé humaine des organismes vivants afin de guider les évaluations des risques.
- Assurent que les organismes formés de molécules d'ADN recombiné sont évalués pour déterminer les risques possibles, préalablement à leur application dans l'agriculture et dans l'environnement par un examen distinct des risques potentiels de façon ponctuelle.
- Dirigent le développement d'organismes formés d'ADN recombiné pour des applications agricoles et environnementales d'une manière progressive, allant si approprié, du laboratoire à la chambre de culture et à la serre, puis à des essais limités en conditions réelles, et finalement à des essais au champ à grande échelle.
- Encouragent la recherche pour améliorer les prédictions, l'évaluation et le suivi des résultats des applications d'organismes formés d'ADN recombiné. »

#### *Rôle du confinement dans les essais à échelle réduite*

En 1992, l'OCDE a publié son Principe d'élaboration saine (OCDE, 1992) pour la conception de recherche sur le terrain à échelle réduite impliquant des végétaux et microorganismes GM. Ce document, entre autres, décrit l'utilisation du *confinement* dans les tests sur le terrain. Le confinement comprend des mesures pour éviter la dissémination ou l'établissement des organismes d'un terrain faisant l'objet d'une étude, comme par exemple l'isolation physique, temporelle ou biologique (comme l'utilisation de la stérilité).

### ***Mise à l'échelle des végétaux cultivés – « analyse des risques et de la sécurité »***

À partir de 1993, l'attention a été transférée à la mise à l'échelle des végétaux cultivés au fur et à mesure que les sélectionneurs de végétaux commençaient à accroître la production et la commercialisation des végétaux GM. L'OCDE a publié les principes généraux pour la mise à l'échelle (OCDE, 1993a), lesquels réaffirmaient que, « *La sécurité en biotechnologie est réalisée par l'application appropriée de l'analyse des risques et de la sécurité et de la gestion des risques. L'analyse des risques et de la sécurité comprend l'identification des dangers et, si un danger a été identifié, la gestion du risque. L'analyse des risques et de la sécurité est fondée sur les caractéristiques de l'organisme, le trait caractéristique introduit, l'environnement dans lequel l'organisme est introduit, les interactions entre l'environnement et l'organisme de même que l'application prévue. L'analyse des risques et de la sécurité est menée préalablement à une action visée et est en général une composante de routine de la recherche, du développement et des essais de nouveaux organismes, que ces actions soient effectuées en laboratoire ou sur le terrain. L'analyse des risques et de la sécurité est une procédure scientifique qui n'implique ni n'exclut une surveillance au niveau de la réglementation, et qui n'exige pas que chaque cas soit nécessairement examiné par une autorité nationale ou autre* » (OCDE, 1993)a.

### ***Rôle de la familiarité dans l'évaluation des risques et de la sécurité***

La question de la mise à l'échelle a également mené à un concept important, la *familiarité*, qui constitue l'une des approches stratégiques utilisées par la suite pour aborder la sécurité environnementale des végétaux transgéniques.

Le concept de la familiarité est basé sur le fait que la plupart des organismes transformés génétiquement sont développés à partir d'organismes comme les végétaux cultivés, dont la biologie est bien comprise. Elle ne constitue pas une évaluation des risques et de la sécurité en elle-même (NAS, 1989). Toutefois, le concept facilite les évaluations des risques et de la sécurité parce que la familiarité suppose que l'on dispose de suffisamment de renseignements pour être en mesure de poser un jugement sur la sécurité ou sur le risque (U.S. NAS, 1989). La familiarité peut aussi être utilisée pour indiquer les pratiques de gestion appropriées, comme par exemple déterminer si les pratiques agricoles standard sont adéquates ou si d'autres pratiques de gestion sont nécessaires pour gérer le risque (OCDE, 1993a). La familiarité permet à l'évaluateur de risques d'appliquer ses connaissances et son expérience de l'introduction des végétaux et des microorganismes dans l'environnement, ce qui lui donne une indication des pratiques de gestion appropriées. Comme la familiarité dépend aussi de la connaissance de l'environnement et de ses interactions avec les organismes introduits, l'évaluation des risques et de la sécurité effectuée dans un pays peut ne pas s'appliquer à un autre pays. Toutefois, au fur et à mesure que les essais en champ sont effectués, des renseignements sur les organismes impliqués de même que sur leurs interactions avec divers environnements seront recueillis.

La familiarité provient des connaissances et de l'expérience disponibles pour analyser les risques et la sécurité préalablement à la mise à l'échelle de toute nouvelle lignée de végétaux ou cultivars dans un environnement particulier. Pour les végétaux par exemple, la familiarité tient compte, sans y être limitée, des connaissances et de l'expérience au niveau :

- « Des végétaux cultivés, y compris leurs caractéristiques de floraison et de reproduction, leurs exigences écologiques et les expériences passées en matière de sélection des végétaux.
- De l'environnement agricole et environnant du site d'essais.
- Du ou des trait(s) caractéristique(s) spécifique(s) transféré(s) à la ou les lignée(s) de végétaux.

- Des résultats des précédents travaux de recherche fondamentale, notamment la recherche en serre et à échelle réduite sur la nouvelle lignée de végétaux ou sur d'autres lignées présentant les mêmes traits caractéristiques.
- De la mise à l'échelle de lignées de végétaux cultivés développés par des techniques plus traditionnelles de sélection des végétaux.
- De la mise à l'échelle d'autres lignées de végétaux développées par la même technique.
- De la présence de végétaux apparentés (et sexuellement compatibles) dans l'environnement naturel et des connaissances au niveau de la possibilité de transfert génique entre la plante cultivée et la plante apparenté.
- Des interactions entre la plante cultivée, l'environnement et les traits caractéristiques et des interactions au sein de la plante cultivée. » (OCDE, 1993a).

#### *Évaluation des risques et de la sécurité et gestion des risques*

L'évaluation des risques et de la sécurité suppose l'identification des effets nocifs ou des dangers possibles au niveau de l'environnement et la détermination, lorsqu'un danger est identifié, de la probabilité qu'il se produise. Si un danger ou un effet nocif sur la santé est identifié, des mesures doivent être entreprises pour le minimiser ou l'atténuer. C'est ce que l'on appelle la gestion des risques. La certitude absolue ou l'absence totale de risques est impossible à obtenir en matière d'évaluation de la sécurité. L'incertitude est donc un aspect inévitable de toutes les évaluations des risques et de toute gestion des risques (OCDE, 1993a). Par exemple, l'on retrouve de l'incertitude en extrapolant les résultats des tests effectués sur une espèce pour identifier les effets possibles chez une autre espèce. Les évaluateurs et les gestionnaires de risques déploient donc des efforts considérables à traiter les incertitudes. Plusieurs des activités des organisations gouvernementales, comme l'OCDE, tentent de déterminer des façons de gérer ces incertitudes (OCDE 2000).

## ANNEXE II

### Références (par ordre chronologique)

- Considérations de sécurité relatives à l'ADN recombiné. Considérations de sécurité relatives à l'utilisation d'organismes obtenus par des techniques de recombinaison de l'ADN dans l'industrie, dans l'agriculture et dans l'environnement (« *Livre bleu* »), OCDE, 1986.
- Field Testing of Genetically Modified Organisms: Framework for Decisions. U.S. NAS - National Academy of Sciences, National Academy Press, Washington DC. Etats-Unis 1989.
- Bons principes de développement (BPD), OCDE 1992.
- Considérations de sécurité relatives à la biotechnologie : Passage à l'échelle supérieure des plantes cultivées, OCDE, 1993a.
- Méthodes traditionnelles de sélection des plantes : Un aperçu historique destiné à servir de référence pour l'évaluation du rôle de la biotechnologie moderne, OCDE 1993b.
- Commercialisation of Agricultural Products Derived through Modern Biotechnology: Survey Results, OCDE 1995a.
- Report of the OECD Workshop on the Commercialisation of Agricultural Products Derived through Modern Biotechnology, OCDE 1995b.
- Analysis of Information Elements Used in the Assessment of Certain Products of Modern Biotechnology, OCDE 1995c.
- Industrial Products of Modern Biotechnology Intended for Release to the Environment: The Proceedings of the Fribourg Workshop, OCDE 1996.
- Rapport du Sous-groupe sur l'harmonisation de la surveillance réglementaire en biotechnologie au Sommet du G8 d'Okinawa, OCDE 2000.

*PART I*  
**CONSENSUS DOCUMENTS ON THE BIOLOGY OF CROPS**

## SECTION I

### POINTS TO CONSIDER FOR CONSENSUS DOCUMENTS ON THE BIOLOGY OF CULTIVATED PLANTS

*As most of the Consensus Documents included in this book were published before the "Points to Consider" were drafted, some do not follow closely the recommendations in this text.*

#### Introduction

Most environmental risk/safety assessments of transformed (genetically modified or engineered) plants are based upon a broad body of knowledge and experience with the untransformed species (variety, etc.), i.e. familiarity with the crop plant. The intent of the biology consensus document is to describe portions of this body of knowledge directly relevant to risk/safety assessment in a format readily accessible to regulators. The document is not an environmental risk/safety assessment of the species. Rather, the consensus document provides an overview of pertinent biological information on the untransformed species to help define the baseline and scope (the comparator against which transformed organisms will be compared), in the risk/safety assessment of the transformed organism. Consensus documents are not detailed crop handbooks or manuals of agricultural or silvicultural practice or economic botany, but rather focus on the biological information and data that may be clearly relevant to the assessment of newly transformed plants.

This *Points to Consider* document is meant as a structured explanatory checklist, regarding both order and contents, of relevant points to consider in preparing or evaluating a consensus document on the biology of a cultivated vascular plant species or other taxonomic group of interest, in relation to biotechnology and environmental risk/safety assessment. The general approach laid out in this document may also be pertinent to non-vascular plants (e.g. mosses), and fungi and micro-organisms; however, these groups are biologically and ecologically so different that further adaptation and refinement of the general approach will be necessary.

The biology consensus documents that have been published to date as well as most in preparation [excepting the documents on *Pleurotus* spp. (oyster mushrooms) and several on micro-organisms] are on annual crops, timber trees, and fruit trees. The plants of interest that have been the subject of the documents are primarily row crops, or trees managed silviculturally or grown in plantations or orchards. They are vascular plants, either flowering plants (angiosperms) or conifers (gymnosperms).

The points to consider as covered in the present document create a basic format and scope to be used for writing or reviewing new consensus documents and updating the earlier documents. While this *Points to Consider* document is meant to provide a basic format and scope, it is not intended to be rigid or inflexible. Of the biology consensus documents to date, some have addressed a particular point in depth, others lightly, and some not at all, depending on the relevance of the point to the plant species or other group of interest. Should additional points beyond those covered in this document be needed for a particular plant, the additional information can be included in the body of the consensus document, or in appendices. If a particular point is not covered in a consensus document, the text may briefly explain why the point, in the particular case, is not relevant.

Authors of the draft of a plant biology consensus document should be familiar with this *Points to Consider* document as well as existing consensus documents in the OECD Series on Harmonisation of Regulatory Oversight in Biotechnology (SHROB), in order to develop the appropriate scoping and presentation of information and data and for general editorial style. Existing consensus documents, particularly more recent ones, may provide detailed examples (some noted below) that are helpful models or thought-provoking for particular cases. Those interested in gaining perspective on coverage of pertinent topics as these documents have evolved and been fine-tuned for the varied cultivated plants in relation to environmental risk/safety assessment may consult especially *Analysis of Consensus Document's Section I: Analysis and Comparison of Consensus Documents* [ENV/JM/BIO(2003)16]. This review was presented at the OECD Workshop on Review of Consensus Documents and Future Work in Harmonisation, held in Washington, D.C., USA in October 2003. This document on points to consider for consensus documents on the biology of cultivated plants results from a recommendation of that meeting.

An understanding of the biology of the species or other group of interest will aid in determining the kinds of information pertinent to the environmental risk/safety assessment. This *Points to Consider* document provides an explanation of why the point (as enumerated below) is important in risk/safety assessment of the transformed plant, and presents a rationale for how the information in the point relates to risk/safety assessment. For a particular environmental risk/safety assessment, biological or ecological information in addition to that presented in the consensus document may be needed to address the regional environments into which the genetically engineered plant is proposed to be released.

## 1. Species or Taxonomic Group

The focus of each biology consensus document has usually been a species, but in some cases the focus has been a group of species or a genus, or just a subspecies or a cultivar group (examples are below). The primary focus of this *Points to Consider* document also is the species of interest, so appropriate adjustments will be necessary if the focus of the consensus document is more broad or narrow.

### 1.1. Classification and nomenclature

Give the scientific name of the cultivated species of interest, with its authors, and pertinent synonyms (*i.e.* actively used alternative scientific names, if any). If necessary to delimit the plant, also give the horticultural name, *e.g.* the cultivar group (*e.g.* *Beta vulgaris* subsp. *vulgaris* Sugar Beet Group). Provide main international common name(s) at least in English for the species of interest. Give the taxonomic context of the species (family always, perhaps the order, and perhaps the subfamily, tribe, subgenus or section). If the taxonomy is not settled, be relatively conservative in choosing the taxonomy, and briefly explain the alternative(s). The latest taxonomic or nomenclatural study is not necessarily definitive, and may need time for scientific consensus before it becomes adopted. A common name for the crop species of interest can be introduced here, to be used in much of the document as a more familiar name (*aide-memoire*).

Describe the taxonomic relationships of the cultivated species: related species, and related genera particularly if there is good potential for spontaneous hybridisation or the generic limits are unsettled. A list of related species (with brief ranges) should be given and include all the relatives with a potential for hybridization (*i.e.* crossable relatives). This topic is dealt with in detail in Section IV. The listing here may provide brief information on chromosome numbers and ploidy if these data are pertinent to the taxonomic differentiation of the species, whereas a more complete coverage of the relevant details is provided in Section III or IV.

**Rationale:** The scientific name enables an unequivocal understanding (*i.e.* a circumscription) of the plant of interest, at the appropriate level, such as the species or the subspecies. This addresses what the

species (or other group) is and what it is called (*i.e.* circumscription and name). The list of close relatives could help in subsequent analysis to form an idea of the kinds of pertinent traits such as disease resistance or stress tolerance that may already occur in these direct relatives of the cultivated plant, and may help elucidate how genes/traits are shared and may move via gene flow amongst related populations. The list of close relatives may aid in understanding the range of diversity and variability between the species and its naturally crossable relatives.

**Examples:** OECD Series on Harmonisation of Regulatory Oversight in Biotechnology (SHROB) No. 14 (rice, Section II, pp. 12-14); No. 16 (poplars, Section II, pp. 15-18); No. 18 (sugar beet, Section I, pp. 11-12); No. 22 (eastern white pine, Section II, p. 12); and No. 31 (sunflower, Section I, pp. 11-13).

## 1.2. Description

Give a brief non-technical description of the species of interest, understandable to the non-specialist. Provide the habit and general characteristics of the plant, for example that it is an annual, a long-lived tree, or a biennial cultivated as an annual crop, and that it is, for instance, grown for fibre, fruit, or seeds. Also provide a concise technical (taxonomic) description sufficient to make a positive identification of the plant (or part). Illustration (a line drawing or black-and-white photo) may be useful. To clarify distinctiveness, emphasise the practical diagnostic or distinguishing morphological or other characters. Limit jargon, by the precise use of phrases and familiar words. A table of main differences or taxonomic key may be instructive (*e.g.* *Oryza sativa* and *O. glaberrima* in SHROB No. 14). If necessary, for example when based on recent information or a new approach, present or reference the analytical methods by which a differential identification of the similar plants (*e.g.* species) is now made.

**Rationale:** These descriptions provide broad orientation, and as well accurate identification. They briefly explain how the species of interest is actually identified in relation to others. Additionally, the description may give particular characteristics of the plant to aid in defining the scope of a risk/safety assessment. Although an exact identification often is based on experience (*i.e.* recognition) or on regional publications, rigorous or subtle analysis using specialist resources sometimes is required.

**Examples:** OECD SHROB No. 8 (potato, Section IV, pp. 14-15) and No. 28 (European white birch, Section I, pp. 12-13).

## 1.3. Geographic distribution, natural and managed ecosystems and habitats, cultivation and management practices, and centres of origin and diversity

This subsection covers the primary or crop species of interest, including the plants that are wild or free-living (whether native or naturalised) or weedy, and as cultivated or managed in the field. Crossable relatives with the relevant information and data on their intraspecific and interspecific crossing are discussed in Sections III and IV.

### 1.3.1. Geographic distribution

Describe the overall geographic distribution (if helpful including altitudinal range or climatic region), indicating broadly where the species of interest is native (*i.e.* indigenous), where it has been naturalised (introduced but free-living), and where it is in cultivation. A general map may be useful.

**Rationale:** Knowledge of the geographic distribution sets the context for understanding the potential interaction of the species with its relatives and with the surrounding ecosystems. For example, it is important to make a distinction between the species' native and naturalised occurrence when assessing the potential effects and the importance of gene flow.

**Examples:** OECD SHROB No. 8 (potato, Sections II & III, pp. 12-13); No. 13 (white spruce, Section III, pp. 15-16); and No. 16 (poplars, Section II, pp. 15-18).

### 1.3.2. Ecosystems and habitats where the species occurs natively, and where it has naturalised

Indicate the natural and non-cultivated or non-managed ecosystems where populations of the species of interest are native (indigenous) and where introduced and now naturalised (free-living) components of the vegetation. Designated natural areas (*e.g.* protected reserves, parks) where the species may be an invasive problem would be noted here. A species weedy in disturbed waste (*e.g.* abandoned) areas would be included here, whereas the species weedy in intensively managed areas would be discussed in the following subsection. Those ecosystems and habitats in which the species of interest occurs and its abundance are indicated here, whereas its ecological interactions with biotic components of the ecosystems and habitats are developed in Section V.

**Rationale:** The focus of this subsection is the relatively natural, self-sustaining context, rather than the land areas strongly managed for plant production. Knowledge of where the species occurs indigenously or is free-living provides baseline information for understanding the range of habitats in which the species exists, the range of behaviours exhibited in those habitats, and how characteristics of the species determine the range of habitats where it occurs. This information provides an understanding of the species' potential for interaction with its relatives and surrounding habitats.

**Example:** OECD SHROB No. 28 (European white birch, Section III, pp. 19-20).

### 1.3.3. Agronomic, silvicultural, and other intensively managed ecosystems where the species is grown or occurs on its own, including management practices

Describe where the species is dependent on management for survival or persistence over several years of usual conditions. Areas where the plant may be a weed problem would be discussed here. Areas to be discussed could include habitats such as annual row crops or bordering areas, tree plantations, orchards and vineyards, along regularly managed roadsides, rights-of-way, irrigation ditches, etc. Identify the pertinent general agronomic or other practices, and if relevant, regional differences in practices (including various practices within a region). Information might briefly encompass site preparation after clear-cutting, tillage, sowing or planting, weed control, control of volunteers, harvesting, plant protection practices during crop growth and after harvest, transport practices, and the use of harvested materials (*e.g.* for silage). The relevant ecological interactions of the species with particular organisms in these managed ecosystems are discussed in Section V.

**Rationale:** The focus of this subsection is on the plant's survival in agro-ecological, silvicultural, and other such managed areas, to provide the baseline environmental information on how the plant responds to or is managed by accepted agronomic, silvicultural or similar intensive practices. Identification of significant cultivation or management practices provides an understanding of measures available to manage or control the plant.

**Examples:** OECD SHROB No. 7 (oilseed rape, Section III, p. 13); No. 14 (rice, Section VII, pp. 26-27); No. 15 (soybean, Sections II & V, pp. 13 & 14); and No. 18 (sugar beet, Sections I & II, pp. 16-17).

### 1.3.4. Centres of origin and diversity

Describe the known or probable primary centre(s) of origin, as well as secondary centres where additional important variability or biodiversity may occur, whether naturally (*e.g.* *Beta*) or through the process of domestication (*e.g.* *Zea mays*, *Solanum tuberosum* subsp. *tuberosum*). The evolutionary centres important for natural biodiversity should be mentioned, and the central areas of domestication and landrace

diversity, with indication of the centres' relative importance. Genetic diversity is covered in Section III. Provide a brief sketch of the history or extent of domestication including mention of relevant domestication traits (*e.g.* non-shattering, loss of seed dormancy).

**Rationale:** The interaction of the cultivated plant with close relatives especially in a centre of origin is an important consideration because gene flow, varietal competition, or a change in cultivation practices may alter this especially rich and valuable diversity. If the plant is not expected to be grown near a center of diversity, the absence of such relatives would also be important. A brief review of domestication may provide insight showing the continuity of modification of the species and the degree of the crop plant's adaptation to or dependence on the managed environment.

**Examples:** OECD SHROB No. 9 (bread wheat, Section III, pp. 13-16); No. 27 (maize, Section IV, pp. 18-20); and No. 31 (sunflower, Section I, pp. 14-15).

## **2. Reproductive Biology**

### **2.1. Generation time and duration under natural circumstances, and where grown or managed**

Important aspects of generation time and duration include the time to first flowering and total life cycle of the plant, and time from planting to plow-down. Include the effects of agronomic, silvicultural, and similar practices when describing generation time and duration of the cultivated plant. Important differences within both the natural and the cultivated regions should be noted.

**Rationale:** The generation time and duration are indications of the terms in which environmental effects may occur. Precocious generation times and shorter durations in agriculture affect the likelihood of outcrossing with free-living (wild) relatives, and give a general indication of when outcrossing may first occur.

**Examples:** OECD SHROB No. 14 (rice, Sections V & VII, pp. 21 & 26-27) and No. 18 (sugar beet, Section I, pp. 13-14).

### **2.2. Reproduction (production of flowers or cones, fruits, seeds, and vegetative propagules)**

Include a characterisation of the key stages in the life cycle necessary for the plant to survive, reproduce, and disperse. Particular attention is given to any uncommon survival structures or strategies and their importance under natural and cultivation conditions, and to the dependence of survival and reproduction on ecological and geographical factors.

**Rationale:** The reproductive capabilities of a plant determine the means by which the plant can produce progeny and spread or disperse. Both the plant and its progeny may affect the environment, including other organisms, and thus the time frame and geographic area over which effects might occur.

#### **2.2.1. Floral biology**

Describe the general floral dynamics (*e.g.* flowering season, flowering time, anthesis, selfing and/or outcrossing). Relevant genetic details of the outcrossing and/or selfing are addressed in Section III.

**Rationale:** This information will assist in understanding some of the factors that affect the potential for gene flow, and in assessing particular management strategies for reducing gene flow when outcrossing may occur. Such management strategies may include induced male sterility or asynchronous flowering times.

**Examples:** OECD SHROB No. 8 (potato, Section VI, p. 17); No. 14 (rice, Section V, p. 21); and No. 21 (Sitka spruce, Section III, p. 15).

### **2.2.2. Pollination (wind, insects, both, etc.), pollen dispersal, pollen viability**

Describe observed modes of pollen dispersal, indicating the most prevalent way. Important insect or other animal pollinators should be indicated. Give data on the range of pollen dispersal through the air and/or by the animal vectors, if known. Note how climatic or regional (*e.g.* geographic) differences can affect pollination. Provide available information or data on the influence of pollen quantity, movement, viability, load and competition on outcrossing, which is discussed in Sections III and IV. The details on pollination as they pertain to the plant are covered here, whereas details particularly pertinent to the pollinator are covered in Section V.

**Rationale:** Pollen biology is an important component in the assessment of potential for gene flow, and in the evaluation of a need for and the type(s) of pollen confinement strategies such as buffer rows or isolation distances.

**Examples:** OECD SHROB No. 8 (potato, Section VI, p. 17) and No. 18 (sugar beet, Section IV, pp. 22-23).

### **2.2.3. Seed production, and natural dispersal of fruits, cones, and/or seeds**

Briefly describe the sexual reproductive structures, including relevant morphological characteristics of fruits (or cones) and seeds, and note any inherent means of dispersal (*e.g.* shattering, fruit splitting, ballistic). Note the quantity of seeds produced by a plant (*e.g.* seeds per fruit and number of fruits). Provide information on the means and range of dispersal (*e.g.* by gravity, wind, water, on and/or in animals), and if there are several means indicate their relative importance. Cover apomixis below, in Subsection 2.2.5.

**Rationale:** The number of seeds and seed/fruit dispersal mechanisms are factors to consider in understanding the potential for establishment of free-living plants or populations, and thus the time and geographic area over which environmental effects might occur. The range of variability of these factors is also an important consideration.

**Examples:** OECD SHROB No. 15 (soybean, Section IV, p. 14) and No. 28 (European white birch, Section IV, p. 23).

### **2.2.4. Seed viability, longevity and dormancy, natural seed bank; germination, and seedling viability and establishment**

Discuss factors in the establishment of any seed bank, including its transience or persistence, and the viability, longevity and dormancy of seeds under natural conditions. Note any special conditions that affect dormancy and/or germination (*e.g.* depth of burial, light and/or temperature, passage through an animal's digestive tract, or need for fire) that might be particularly relevant. Note any special requirements for the establishment and survival of seedlings (*e.g.* soil qualities or regime), as the organism's fitness may be revealed at this challenging phase in the life cycle.

**Rationale:** Seed viability is a key factor to consider in assessing the likelihood of survival of non-cultivated plants. Natural seed banks are often the main source of weeds in cultivated fields, whether they are previous-crop volunteers or non-crop weedy relatives. Whether seedlings can establish usually is a primary limiting factor in continuing the life cycle.

**Example:** OECD SHROB No. 7 (oilseed rape, Section VI, p. 17).

### 2.2.5. Asexual propagation (apomixis, vegetative reproduction)

Take into account natural vegetative cloning (*e.g.* in grasses and poplars), the kinds of propagules (special structures, and/or fragmented plant pieces), dispersal of the propagules, and their viability. Discuss the relative importance of asexual reproduction for the plant, including any differences dependent on habitat or region. For apomixis (non-sexual production of seeds), similarly consider its relative importance and effectiveness.

**Rationale:** If a plant has a strategy that includes asexual propagation, this could be a means for considerable or quite different dispersal or spread, and consequently may also affect the time frame and geographic area over which environmental effects might occur.

**Example:** OECD SHROB No. 16 (poplars, Section IV, p. 23).

## 3. Genetics

### 3.1. Relevant detailed genetic information on the species

Give a basic overview of the relevant genetic constitution and genetic dynamics of the species. If more appropriate in a particular case, some basic genetic information (*e.g.* ploidy, ancestral/progenitor genomes) may be more fully or instead discussed in Section IV. In this Section III (including subsections as needed), cover for example and if appropriate cytogenetics (*e.g.* karyology, meiotic behavior), nuclear genome size, possible extent of repetitive or non-coding DNA sequences, main genetic diversity or variability (*e.g.* among or within populations or varieties, and of alleles at a locus), evidence of heterosis or inbreeding depression, maternal and/or paternal inheritance of organellar genomes, and methods of classical breeding (*e.g.* utility from employing mutagenesis with the species). The relevance of the information to the species' variability and the potential effects of transformation are paramount in deciding what to include, as the focus is not to provide this genetic characterisation for plant development.

Intraspecific crossing with both non-cultivated strains (*e.g.* weedy races) and among non-transformed cultivars is appropriately covered here (perhaps with a table or diagram), including any genetic or cytoplasmic constraints or limitations to crossing (*e.g.* cytoplasmic or nuclear sterility, incompatibility systems). Interspecific crosses are addressed in the following section.

**Rationale:** The information in this section includes genetic and breeding data, such as details of genomic or genetic stability (including gene silencing) and intraspecific outcrossing behaviour and potential, only to the extent that such information describes parameters that influence how genetic material (including new material) behaves in particular genetic backgrounds, and in outcrossing. Interspecific hybridisation is in a separate section (which follows) because intraspecific crossing is more likely (and familiar), and interspecific hybrids may bring in broader or more extensive concerns.

**Examples:** OECD SHROB No. 9 (bread wheat, Sections III & V, pp. 13-17 & 20-24); No. 12 (Norway spruce, Section VI, pp. 21-23); No. 13 (white spruce, Section V, pp. 22-24); No. 14 (rice, Section VI, pp. 23-25); No. 24 (*Prunus* spp. – stone fruits, Section II, pp. 15-20); and No. 31 (sunflower, Section IV, pp. 27-28).

## 4. Hybridisation and Introgression

### 4.1. Natural facility of interspecific crossing (extent, sterility/fertility)

Describe interspecific (including intergeneric) crosses observed under natural conditions. Provide a list and perhaps a diagram of the documented hybrids, *i.e.* the crossings that may occur unaided under

usual environmental conditions — if the crossable relatives (other species) might be present. The information could include a discussion of ploidy (and ancestral/progenitor genomes). Provide an indication or review of the likelihood of first-generation ( $F_1$ ) hybrids and later generations of these  $F_1$  hybrids, and as well whether the  $F_1$  hybrids may be bridges for genes to cross into other (non-parental) species. Rare plant species are considered here and in the following subsection. Indicate naturally hybridising species that are weedy (including invasive) in the list of hybridising species (detailed discussion of their weediness in a local environment would be covered in an environmental risk/safety assessment).

**Rationale:** The ability of a cultivated species to hybridise with other cultivated or wild species is a significant factor in determining whether genes or traits could be transferred to other species.

**Examples:** OECD SHROB No. 7 (oilseed rape, Section VII, pp. 18-21); No. 9 (bread wheat, Section V, pp. 20-24); and No. 16 (poplars, Sections III & VI, pp. 20 & 28-29).

#### 4.2. Experimental crosses

Discuss the experimental data available on outcrossing under controlled conditions, and theoretical possibilities for and barriers to outcrossing. This information is in contrast to that in the previous subsection, which indicates the outcrossing to readily crossable relatives. Experimental data that is the result of forced crosses employing special techniques (*e.g.* embryo rescue) would be relevant only if such studies help to clarify degree of relatedness and likelihood of natural crossing. Theoretical considerations or experimental information might be, for example, on cytogenetic data and meiotic behaviour, or sexual incompatibility systems.

**Rationale:** Experimental data and theoretical considerations may broaden the understanding of potential (or as yet unknown) unaided (natural) gene transfer. The information and data are only relevant if unaided crossing in the field can occur.

**Examples:** OECD SHROB No. 8 (potato, Section VII, pp. 19-21); No. 13 (white spruce, Section VI, pp. 25-26); No. 16 (poplars, Section VI, pp. 28-29); and No. 22 (eastern white pine, Section IV, p. 17).

#### 4.3. Information and data on introgression

Provide an indication or review of the likelihood of  $F_1$  hybrids backcrossing into one or both parents. Provide information on both natural and experimental introgression (extensive backcrossing), and on the (types of) genes or the traits for which introgression has been demonstrated. For example, extensive backcrossing and introgression may be only in one direction, rather than into both parental lines or species' populations. Information should include the extent of likely natural (*i.e.* unaided) introgression or generations of experimental backcrossing, and the fertility and fecundity of the resultant plants.

**Rationale:** Of primary consideration is whether interspecific crossing will lead to the introgression of genes. Interspecific crossing is a necessary but typically not a sufficient step for considerable introgression to occur. Even if introgression occurs, it is not the presence but the expression of the gene or trait that may be of primary importance.

**Examples:** OECD SHROB No. 7 (oilseed rape, Section VII, pp. 20-21); No. 24 (*Prunus* spp. – stone fruits, Section II, p. 30); and No. 31 (sunflower, Section IV, pp. 28-29).

## 5. General Interactions with Other Organisms (Ecology)

### 5.1. Interactions in natural ecosystems, and in agronomic, silvicultural or other ecosystems where the species is cultivated or managed

Provide a general overview (including subsections as needed) of main functional ecological interactions of the species of interest within these natural and managed ecosystems and habitats, for example symbiotic relationships, food webs (*e.g.* fruit and seed consumers or predators), noxious/toxic or other important interactions with insects (*e.g.* chemical defense) and other animals, and with plants (*e.g.* allelopathy). Tritrophic interactions may also be considered. Subsections 1.3.2 and 1.3.3 list and briefly characterise the natural (unmanaged) and managed ecosystems and habitats in which the species of interest occurs. The importance of a pollination system to the animal pollinator is detailed here, whereas the importance to the plant is addressed in Subsection 2.2.2. A listing of pertinent pests and pathogens (and diseases) may be presented as an appendix, with only those that are critically relevant discussed here.

**Rationale:** The description of the basic general ecology of the species of interest is useful when determining the scope of interactions that may be used as a baseline for understanding the influences the cultivated plant may have on organisms that are in usual close contact. A general understanding of the interactions of the species with other organisms will aid in determining whether any concerns may arise with a change in the genetics of the species.

**Examples:** OECD SHROB No. 7 (oilseed rape, Section VII & Appendix, pp. 21 & 29) and No. 13 (white spruce, Section VII, pp. 28-31).

## Human Health and Biosafety

### 6.1. Plant characteristics relevant for human health

Provide brief information on major natural toxicants and common allergenic or medicinal properties of the plant. In some cases, it may be relevant to mention similar information from related species (*e.g.* glycoalkaloids in crossable wild relatives of *Solanum tuberosum* subsp. *tuberosum*, potato).

**Rationale:** This theme can be regarded as human ecology, a subset of Section V that warrants coverage separately. Baseline information is briefly described, relating to human health as it might be affected by cultivation of the plant (*e.g.* levels of latex or psoralen). Potential effects on human health would be thoroughly treated elsewhere, such as in an OECD plant compositional consensus document for dietary issues.

**Example:** OECD SHROB No. 8 (potato, Section IV, p. 14).

## 7. Additional Information

The possibility is expressly left open for topics of additional information that is pertinent to environmental risk/safety assessment, as a section in the main text of the document, and/or as appendices.

## 8. References

As much as possible, the references should be peer-reviewed literature available internationally. After the references directly cited in the text, this section could include a subsection on additional useful references 'for further reading'.

**Example:** OECD SHROB No. 7 (oilseed rape, Section IX, pp. 27-28).

## **Appendix I – Common Pests and Pathogens**

Provide a list of causative organisms for diseases (pathogens) and pests that commonly occur in the crop under agronomic, silvicultural, or equivalent conditions.

**Rationale:** Provide as considered useful for risk/safety assessment rather than usual production management. Critically important organisms and ecological relationships (*e.g.* a virus disease that is a principal management issue) are covered in Section V. The risk/safety assessment would then consider whether the transformation in the crop would be of environmental concern.

**Examples:** OECD SHROB No. 18 (sugar beet, Appendix, pp. 32-37 and No. 31 (sunflower, Section V & Appendices 1 & 2, pp. 31 & 37-47).

## **Appendix II – Biotechnological Developments**

General information on the kinds of traits being introduced into the species may be included. Provide information directly necessary for defining the scope or detail of biological information that would be useful. For example, transgenes under experimental development for a crop might result in a change in environmental fitness or range and habitats of the plant or its relatives (*e.g.* disease resistance, and drought, frost or salinity tolerance). Other biotechnological developments (*e.g.* to assist in marketing) may not be pertinent to address here.

**Rationale:** An overview of biotechnological developments may help to assure that the biological information included in a consensus document is pertinent to the environmental risk/safety assessments anticipated. Consensus documents that include the biotechnological developments to bring traits into the crop can be quite useful in explaining the relevance of assessing certain kinds of biosafety information.

**Examples:** OECD SHROB No. 14 (rice, Appendix III, pp. 42-45) and No. 27 (maize, Appendix A, pp. 39-41).

## SECTION 2

### SOYBEAN (*GLYCINE MAX* (L.) MARR.)

#### 1. General Description Including Taxonomy and Morphology, and Use as a Crop Plant

Cultivated soybean, *Glycine max* (L.) Merr., is a diploidized tetraploid ( $2n=40$ ), in the family Leguminosae, the subfamily Papilionoideae, the tribe Phaseoleae, the genus *Glycine* Willd. and the subgenus *Soja* (Moench). It is an erect, bushy herbaceous annual that can reach a height of 1.5 metres. Three types of growth habit can be found amongst soybean cultivars: determinate, semi-determinate and indeterminate (Bernard and Weiss, 1973). Determinate growth is characterised by the cessation of vegetative activity of the terminal bud when it becomes an inflorescence at both axillary and terminal racemes. Determinate genotypes are primarily grown in the southern United States (Maturity Groups V to X). Indeterminate genotypes continue vegetative activity throughout the flowering period and are grown primarily in central and northern regions of North America (Maturity Groups 000 to IV). Semi-determinate types have indeterminate stems that terminate vegetative growth abruptly after the flowering period. None of the soybean varieties are frost tolerant, and they do not survive freezing winter conditions.

The primary leaves are unifoliate, opposite and ovate, the secondary leaves are trifoliate and alternate, and compound leaves with four or more leaflets are occasionally present. The nodulated root system consists of a taproot from which emerges a lateral root system. The plants of most cultivars are covered with fine trichomes, but glabrous types also exist. The papilionaceous flower consists of a tubular calyx of five sepals, a corolla of five petals (one banner, two wings and two keels), one pistil and nine fused stamens with a single separate posterior stamen. The stamens form a ring at the base of the stigma and elongate one day before pollination, at which time the elevated anthers form a ring around the stigma. The pod is straight or slightly curved, varies in length from two to seven centimetres, and consists of two halves of a single carpel which are joined by a dorsal and ventral suture. The shape of the seed, usually oval, can vary amongst cultivars from almost spherical to elongate and flattened.

Soybean is grown as a commercial crop in over 35 countries. The major producers of soybeans are the United States, China, Democratic People's Republic of Korea and Republic of Korea, Argentina and Brazil. Soybean is grown primarily for the production of seed, has a multitude of uses in the food and industrial sectors, and represents one of the major sources of edible vegetable oil and of proteins for livestock feed use.

A major food use in North America and Europe is as purified oil, utilised in margarines, shortenings and cooking and salad oils. It is also used in various food products, including tofu, soya sauce, simulated milk and meat products. Soybean meal is used as a supplement in feed rations for livestock. Industrial use of soybeans ranges from the production of yeasts and antibodies to the manufacture of soaps and disinfectants.

Soybean is commonly considered one of the oldest cultivated crops, native to North and Central China (Hymowitz, 1970). The first recording of soybeans was in a series of books known as Pen Ts'ao Kong Mu written by the emperor Sheng Nung in the year 2838 B.C., in which the various plants of China are described. Historical and geographical evidence suggests that soybeans were first domesticated in the

eastern half of China between the 17th and 11th century B.C. (Hymowitz, 1970). Soybeans were first introduced into the United States, now a major producer, in 1765 (Hymowitz and Harlan, 1983).

## 2. Agronomic Practices

Soybean is a quantitative short day plant and hence flowers more quickly under short days (Garner and Allard, 1920). As a result, photoperiodism and temperature response is important in determining areas of cultivar adaptation. Soybean cultivars are identified based on bands of adaptation that run east-west, determined by latitude and day length. In North America, there are thirteen maturity groups (MG), from MG 000 in the north (45° latitude) to MG X near the equator. Within each maturity group, cultivars are described as early, medium or late maturing.

The seed will germinate when the soil temperature reaches 10°C and will emerge in a 5-7 day period under favourable conditions. In new areas of soybean production an inoculation with *Bradyrhizobium japonicum* is necessary, for optimum efficiency of the nodulated root system. Soybeans do not yield well on acid soils and the addition of limestone may be required. Soybeans are often rotated with such crops as corn, winter wheat, spring cereals, and dry beans.

## 3. Centres of Origin of the Species

*Glycine max* belongs to the subgenus *Soja*, which also contains *G. soja* and *G. gracilis*. *Glycine soja*, a wild species of soybean, grows in fields, hedgerows, roadsides and riverbanks in many Asian countries. Wild soybean species are endemic in China, Korea, Japan, Taiwan and the former USSR, but do not exist naturally in North America. Cytological, morphological and molecular evidence suggest that *G. soja* is the ancestor of *G. max*. *Glycine gracilis* is considered to be a weedy or semi-wild form of *G. max*, with some phenotypic characteristics intermediate to those of *G. max* and *G. soja*. *Glycine gracilis* may be an intermediate in the speciation of *G. max* from *G. soja* (Fekuda, 1933) or a hybrid between *G. soja* and *G. max* (Hymowitz, 1970).

## 4. Reproductive Biology

Soybean is considered a self-pollinated species, propagated commercially by seed. Artificial hybridisation is used for cultivar breeding.

The soybean flower stigma is receptive to pollen approximately 24 hours before anthesis and remains receptive 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. As a result, soybeans exhibit a high percentage of self-fertilisation, and cross pollination is usually less than one percent (Caviness, 1966).

A soybean plant can produce as many as 400 pods, with two to twenty pods at a single node. Each pod contains one to five seeds. Neither the seedpod, nor the seed, has morphological characteristics that would encourage animal transportation.

## 5. Cultivated *Glycine max* as a Volunteer Weed

Cultivated soybean seed rarely displays any dormancy characteristics and only under certain environmental conditions grows as a volunteer in the year following cultivation. If this should occur, volunteers do not compete well with the succeeding crop, and can easily be controlled mechanically or chemically. The soybean plant is not weedy in character. In North America, *Glycine max* is not found outside of cultivation. In managed ecosystems, soybean does not effectively compete with other cultivated plants or primary colonisers.

## 6. Crosses

### A. Inter-species/genus

In considering the potential environmental impact following the unconfined release of genetically modified *Glycine max*, it is important to have an understanding of the possible development of hybrids through interspecific and intergeneric crosses with related species. The development of hybrids could result in the introgression of the novel traits into these related species and result in:

- The related species becoming more weedy.
- The introduction of a novel trait, with potential for ecosystem disruption, into related species.

For a trait to become incorporated into a species genome, recurrent backcrossing of plants of that species by hybrid intermediaries, and survival and fertility of the resulting offspring, is necessary.

The subgenus *Soja*, to which *G. max* belongs, also includes *G. soja* Sieb. and Zucc. ( $2n=40$ ) and *G. gracilis* Skvortz. ( $2n=40$ ), wild and semi-wild annual soybean relatives from Asia. *Glycine soja* ( $2n=40$ ) is a wild viny annual with small and narrow trifoliate leaves, purple flowers and small round brown-black seeds. It grows wild in Korea, Taiwan, Japan, Yangtze Valley, N.E. China and areas around the border of the former USSR. *Glycine gracilis*, an intermediate in form between *G. soja* and *G. max*, has been observed in Northeast China (Skvortzow, 1927). Interspecific, fertile hybrids between *G. max* and *G. soja* (Sieb and Zucc.) (Ahmad *et al.*, 1977; Hadley and Hymowitz, 1973; Broich, 1978), and between *G. max* and *G. gracilis* (Karasawa, 1952) have been easily obtained.

In addition to the subgenus *Soja*, the genus *Glycine* contains the subgenus *Glycine*. The subgenus *Glycine* consists of twelve wild perennial species, including *G. clandestina* Wendl., *G. falcata* Benth., *G. latifolia* Benth., *G. latrobeana* Meissn. Benth., *G. canescens* F.J. Herm., *G. tabacina* Labill. Benth., and *G. tomentella* Hayata. These species are indigenous to Australia, South Pacific Islands, China, Papua New Guinea, Philippines, and Taiwan (Hymowitz and Newell, 1981; Hermann, 1962; Newell and Hymowitz, 1978; Grant, 1984; Tindale, 1984, 1986). Hybrids between diploid perennial *Glycine* species show normal meiosis and are fertile.

Early attempts to hybridise annual (subgenus *Soja*) and perennial (subgenus *Glycine*) species were unsuccessful. Although pod development was initiated, these eventually aborted and abscised (Palmer, 1965; Hood and Allen, 1980; Ladizinsky *et al.*, 1979). Intersubgeneric hybrids were later obtained in vitro through embryo rescue, between *G. max* and *G. clandestina* Wendl.; *G. max* and *G. tomentella* Hayata (Singh and Hymowitz, 1985; Singh *et al.*, 1987); and *G. max* and *G. canescens*, using transplanted endosperm as a nurse layer (Broué *et al.*, 1982). In all cases, the progeny of such intersubgeneric hybrids was sterile and obtained with great difficulty.

### B. Introgression into relatives

Soybean can only cross with other members of *Glycine* subgenus *Soja*. The potential for such gene flow is limited by geographic isolation. Wild soybean species are endemic in China, Korea, Japan, Taiwan and the former USSR. These species are not naturalised in North America, and although they could occasionally be grown in research plots, there are no reports of their escape from such plots to unmanaged habitats.

### C. Interactions with other organisms

The table in the Appendix is intended as an identification guide for categories of organisms, which interact with *Glycine max*. This table, representative of North America, is intended to serve as an example only. Environmental safety assessors should, on a country-by-country basis, draw up their own lists as a guide for assessing potential effects of the release of genetically modified plants on interacting organisms in their country.

The intention is not to require comparison data between a plant with novel traits and its *G. max* counterpart(s) for all interactions. Depending on the novel traits, applicants might decide to submit data for only some of the interactions. Sound scientific rationale will be required to justify the decision that data would be irrelevant for the remaining interactions. For example, the applicant might choose not to provide data on the weediness potential of a plant with novel traits if it can be clearly shown that the novel trait will not affect reproductive or survival characteristics of *G. max*, either directly or indirectly. Some of the life forms are listed as categories (*i.e.* pollinators, mycorrhizal fungi, animal browsers, birds, soil microbes, and soil insects). When, because of the novel traits, a concern is perceived for these specific categories, applicants will be required to provide detailed information on interactions with indicator species in each category. Where the impact of a plant with novel traits on another life form (target or non-target organism) is significant, secondary effects may need to be considered.

This section will be revised to include relevant new data as they become available.

### 7. Summary of Ecology of *Glycine max*

*Glycine max* (L.) Merr., the cultivated soybean, is a summer annual herb that has never been found in the wild (Hymanitz, 1970). This domesticate is in fact extremely variable, due primarily to the development of soybean "land races" in East Asia. The subgenus *Soja* contains, in addition to *G. max* and *G. soja*, the form known as *G. gracilis*, a form morphologically intermediate between the two. This is a semi-cultivated or weedy form, and is known only from Northeast China.

*Glycine soja*, considered the ancestor of cultivated soybean, is an annual procumbent or slender twiner that is distributed throughout China, the adjacent areas of the former USSR, Korea, Japan and Taiwan. It grows in fields and hedgerows, along roadsides and riverbanks.

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## APPENDIX

### Examples of Potential Interactions of *G. max* with Other Life Forms During its Life Cycle

Other life forms Common Name	Interaction with <i>G. max</i> Pathogen; Symbiont or Beneficial Organism; Consumer; Gene Transfer Pathogen
Brown spot ( <i>Septoria glycines</i> )	Pathogen
Downy mildew ( <i>Peronospora trifoliorum</i> var. <i>manshurica</i> )	Pathogen
Brown stem rot ( <i>Phialophora gregata</i> or <i>Acremonium strictum</i> )	Pathogen
Phytophthora root and stalk rot ( <i>Phytophthora megasperma</i> )	Pathogen
Stem canker ( <i>Diaporthe phaseolorum</i> var. <i>caulivora</i> )	Pathogen
Rhizoctonia stem and root rot ( <i>Rhizoctonia solani</i> )	Pathogen
Pythium root rot ( <i>Pythium</i> spp.)	Pathogen
Fusarium wilt, blight, and root rot ( <i>Fusarium</i> spp.)	Pathogen
Sclerotinia stem rot ( <i>Sclerotinia sclerotiorum</i> )	Pathogen
Pod and stem blight ( <i>Diaporthe phaseolorum</i> var. <i>sojae</i> )	Pathogen
Bacterial Blight ( <i>Pseudomonas syringae</i> )	Pathogen
Soybean mosaic virus (SMV)	Pathogen
Anthrachnose ( <i>Colletotrichum truncatum</i> )	Pathogen
Purple seed stain ( <i>Cercospora kikuchii</i> )	Pathogen
Powdery mildew ( <i>Microsphaera diffusa</i> )	Pathogen
Root knot ( <i>Meloidogyne</i> spp.)	Pathogen
Spider mite ( <i>Acari: Terranychidae</i> )	Consumer
Soybean cyst nematode ( <i>Heterodera glycines</i> )	Consumer
Soybean looper, white fly ( <i>Lepidopterans</i> )	Consumer
Soil insects	Consumer
Birds	Consumer
Animal browsers	Consumer
Pollinators	Symbiont or Beneficial Organism; Consumer
Mychorrhizal fungi	Symbiont or Beneficial Organism
Soil microbes	Symbiont or Beneficial Organism
Earthworms	Gene Transfer
Other <i>G. max</i>	Symbiont or Beneficial Organism
Others	---

## SECTION 3 MAIZE (*ZEA MAYS* SUBSP. *MAYS*)

### 1. General Information

Maize, or corn, is a member of the *Maydeae* tribe of the grass family, *Poaceae*. It is a robust monoecious annual plant, which requires the help of man to disperse its seeds for propagation and survival. Corn is the most efficient plant for capturing the energy of the sun and converting it into food, it has a great plasticity adapting to extreme and different conditions of humidity, sunlight, altitude, and temperature. It can only be crossed experimentally with the genus *Tripsacum*, however member species of its own genus (teosinte) easily hybridise with it under natural conditions.

This document describes the particular condition of maize and its wild relatives, and the interactions between open-pollinated varieties and teosinte. It refers to the importance of preservation of native germplasm and it focuses on the singular conditions in its centre of origin and diversity. Several biological and socio-economic factors are considered important in the cultivation of maize and its diversity; therefore these are described as well.

#### A. Use as a crop plant

In industrialised countries maize is used for two purposes: 1) to feed animals, directly in the form of grain and forage or sold to the feed industry; and 2) as raw material for extractive industries. "In most industrialised countries, maize has little significance as human food" (Morris, 1998; Galinat, 1988; Shaw, 1988). In the European Union (EU) maize is used as feed as well as raw material for industrial products (Tsaftaris, 1995). Thus, maize breeders in the United States and the EU focus on agronomic traits for its use in the animal feed industry, and on a number of industrial traits such as: high fructose corn syrup, fuel alcohol, starch, glucose, and dextrose (Tsaftaris, 1995). It is also noteworthy to understand how corn is used in the rising consumption of sweet corn and popcorn in developed countries (White and Pollak, 1995; Benson and Pearce, 1987).

In developing countries use of maize is variable; in countries such as Mexico, one of the main uses of maize is for food. In Africa as in Latin America, the people in the sub-Saharan region consume maize as food, and in Asia it is generally used to feed animals (Morris, 1998).

Maize is the basic staple food for the population in many countries of Latin America and an important ingredient in the diet of these people. All parts of the maize plant are used for different purposes: processed grain (dough) to make "tortillas", "tamales" and "tostadas"; grain for "pozole", "pinole" and "pozol"; dry stalks to build fences; a special type of ear cob fungi can be used as food (that is, "corn smut", or *Ustilago maydis*). In general, there are many specific uses of the maize plant depending on the region. Globally, just 21 % of total grain production is consumed as food.

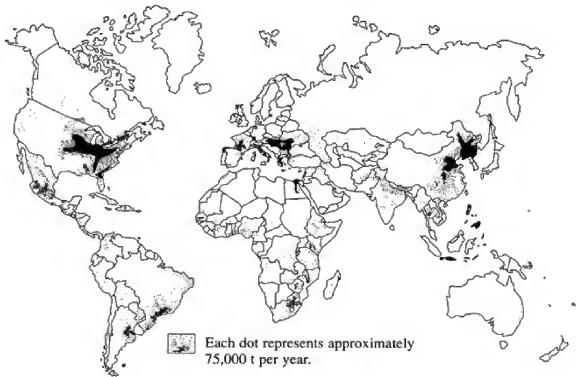
The countries, which have the highest annual maize consumption per capita in the world, are listed in Table 1.1.

Table 1.1 Consumption of maize *per capita* by country

Country	Annual consumption of maize per capita (Kg)
Malawi	137
Mexico	127
Zambia	113
Guatemala	103
Honduras	98
South Africa	94
El Salvador	93
Kenya	93
Zimbabwe	89
Lesotho	87
Venezuela	68
Nicaragua	56

Source : Morris, 1998

Figure 1.1 Maize production worldwide



Source : Morris, 1998

According to Morris (1998), "maize is the world's most widely grown cereal, reflecting its ability to adapt to a wide range of production environments" (Fig. 1).

Transgenic maize is already being used as a crop not only with agricultural purposes in several industrialized countries. Industrialised countries have dominant production of maize, because they possess

advantageous factors that contribute to generate maize surplus. First, "maize production is generally concentrated in zones of abundant rainfall and fertile soils" (Morris, 1998), and, second, the use of many inputs and technology is extensive (Pollak and White, 1995; Rooney and Serna-Saldivar, 1987; Shaw, 1988; White and Pollak, 1995). By contrast, in developing countries the situation is highly variable. From Mexico to the Northern Andean region in South America, maize is a very important staple food in rural areas and the use of technology together with improved varieties is limited. However, Brazil, Argentina and Chile resemble industrialised countries because in these countries maize is a "cash crop grown by large scale commercial producers using extensive mechanisation" (Morris, 1998).

In many countries of Latin America maize is produced on small units of land. For example, in Mexico most of the land planted with maize (77 %) is less than 5 hectares in size, which contributes 67 % of total production (Calva, 1992 in Turrent-Fernández *et al.*, 1997). Only 5 % of the units of land dedicated to the production of maize averaged 12.2 hectares. More recently (Turrent-Fernández *et al.*, 1997), land units of maize production have increased in size but the technology inputs are below average: only 40 % of producers utilised improved seed; 64 % used nitrogen and phosphorous to fertilise the soil; and only 42 % received technical assistance.

In Africa, maize is an important crop mainly in the eastern and southern regions where it is "the dominant food crop and the mainstay of rural diets" (Morris, 1998). Also, maize production in Africa is similar to the production in some Latin American countries because the peasants of less developed rural areas grow maize in small plots, using negligible amounts of inputs or technology and no improved varieties.

In Asia, China dominates maize production. China is the second largest producer of maize closely behind the United States (Morris, 1998). Asian countries produce maize for livestock feed and likewise Meso-America and most African countries; "farms are small, use of improved germplasm and purchased inputs is modest, and yields are generally low" (Morris, 1998).

## 2. Taxonomic Status of *Zea*

The Western Hemisphere genera *Zea* and *Tripsacum* are included in the tribe Maydeae (Table 1.2). The Asian genera of Maydeae are *Coix* ( $2n = 10, 20$ ), *Polytoca* ( $2n = 20$ ), *Chionachne* ( $2n = 20$ ), *Schlerachne* ( $2n = 20$ ) and *Trilobachne* ( $2n = 20$ ).

Based on the morphology of the glumes of the male spikelets, Iltis and Doebley (1980) and Doebley and Iltis (1980) proposed a new classification system of the genus *Zea*. First, *Zea* was separated into two sections: LUXURIANTES and ZEA. The section LUXURIANTES grouped three species: *Z. luxurians*, *Z. diploperennis* and *Z. perennis*, and very recently it has included *Z. nicaraguensis* (Iltis and Benz, 2000). The section ZEA comprises only one species, *Z. mays*, which in turn is sub-divided into three subspecies: ssp. *mays*, for maize, ssp. *mexicana* for the races Nobogame, Central Plateau, Durango and Chalco (Wilkes, 1967; 1977) and ssp. *parviglumis*. This latter in turn is separated into two varieties, var. *parviglumis* for the race Balsas of Wilkes (1967) and var. *huehuetanangensis* for the race Huehuetanango of Wilkes (1967). Later on Doebley (1984, 1990) suggested that the var. *huehuetanangensis* should be elevated to a subspecies level.

Regarding the separation of the genus into sections LUXURIANTES and ZEA there is no controversy since morphological (Doebley, 1983; Smith *et al.*, 1981), isoenzymatic (Doebley *et al.*, 1984; Smith *et al.*, 1984), cytoplasm organelle DNA (Doebley *et al.*, 1987a, b; Sederoff *et al.*, 1981; Timothy *et al.*, 1979), and cytological (Kato, 1984; Kato and Lopez, 1990) evidence supports it.

The main controversy resides on the classification system within the section ZEA, particularly the grouping of the annual teosintes and maize into a single species, *Z. mays*. There is evidence showing that annual teosintes and maize are completely isolated from each other based on chromosome knob data (Kato, 1984; Kato and Lopez, 1990), and morphological-ecological data (Doebley, 1984). Although the isoenzymatic data suggest a low level of introgression between populations of these two plant types (Doebley, 1984; 1990), they have mainly the same isozyme alleles and the frequencies of these are distinct between most of the races of teosinte and most of the races of maize (Goodman, 1988). If it is accepted that the annual teosintes and maize are genetically isolated, then according to the biological species concept, the classification of the section ZEA made by Iltis and Doebley (1980) and Doebley and Iltis (1980) would not be acceptable, and would support the one proposed by Wilkes (1967).

Wilkes (1967) classified the annual teosintes within six races: Nobogame; Central Plateau; Chalco; Balsas; Huehuetenango; and Guatemala. Bird (1978) raised the race Guatemala into species rank, *Z. luxurians*.

The perennial teosintes from Jalisco in Mexico are separated into two more species (Iltis *et al.*, 1979) that have a ploidy difference, *Z. perennis* (2n=40) and *Z. diploperennis* (2n=20).

Doebley and Iltis (1980) and Iltis and Doebley (1980) classified teosinte as two subspecies of *Z. mays*: *mexicana* (Chalco, Central Plateau, and Nobogame) and *parviglumis* (var. *parviglumis*=Balsas and var. *huchuetenangensis*=Huehuetenango).

**Table 1.2 Classification of the genus *Zea* within the tribe Maydeae of the Western Hemisphere, and the genus *Tripsacum***

Family: Poaceae

Subfamily: Panicoideae

Tribe: Maydeae

Western Hemisphere:

Genus *Zea*<sup>1</sup>

Section *ZEA*

*Zea mays* L. (maize)

*Zea mays* subsp. *mays* (L.) Iltis (maize,  $2n^2 = 20$ )

*Zea mays* subsp. *mexicana* (Schrader) Iltis (teosinte,  $2n = 20$ )

    race Nobogame<sup>3</sup>

    race Central Plateau<sup>3</sup>

    race Durango<sup>4</sup>

    race Chalco<sup>5</sup>

*Zea mays* subsp. *parviglumis* Iltis and Doebley (teosinte,  $2n = 20$ )

    var. *parviglumis* Iltis and Doebley (=race Balsas)

    var. *huehuetenangensis* Doebley (=race Huehuetenango)

Section *LUXURIANTES* Doebley and Iltis

*Zea diploperennis* Iltis, Doebley and Guzman (perennial teosinte,  $2n = 20$ )

*Zea luxurians* (Durieu) Bird (teosinte,  $2n = 20$ )

*Zea nicaraguensis*<sup>5</sup> ( $2n = 20?$ )

*Zea perennis* (Hitchc.) Reeves and Mangelsdorf ( $2n = 40$ )

Genus *Tripsacum*

*T. andersonii* ( $2n = 64$ )

*T. australe* ( $2n = 36$ )

*T. bravum* ( $2n = 36, 72$ )

*T. cundinamarce* ( $2n = 36$ )

*T. dactyloides* ( $2n = 72$ )

*T. floridanum* ( $2n = 36$ )

*T. intermedium* ( $2n = 72$ )

*T. manisuioides* ( $2n = 72$ )

*T. latifolium* ( $2n = 36$ )

*T. pereuvianum* ( $2n = 72, 90, 108$ )

*T. zopilotense* ( $2n = 36, 72$ )

*T. jalapense* ( $2n = 72$ )

*T. lanceolatum* ( $2n = 72$ )

*T. laxum* ( $2n = 36?$ )

*T. maizar* ( $2n = 36, 72$ )

*T. pilosum* ( $2n = 72$ )

<sup>1</sup> Iltis and Doebley, 1980; Doebley, 1990. <sup>2</sup> diploidy number. <sup>3</sup> Wilkes, 1967. <sup>4</sup> Sánchez-González *et al.*, 1998. <sup>5</sup> Iltis and Benz, 2000.

### 3. Identification Methods

#### A. General description of *Zea mays*

*Zea mays* is a tall, monoecious annual grass with overlapping sheaths and broad conspicuously distichous blades. Plants have pistillate inflorescences enclosed in numerous large foliaceous bracts (ears), from 7 to 40 cm long, with spikelets in 8 to 16 rows on a thickened axis (cob) in the leaf axils and staminate spikelets in long spike-like racemes that form large spreading terminal panicles (tassels).

#### B. Identification among races of *Zea mays*

To study and classify this huge variation, a system of racial classification was established (Wellhausen *et al.*, 1952; Wellhausen *et al.*, 1957; Brown, 1953; Sato and Yoshida, 1956; Hateway, 1957; Roberts *et al.*, 1957; Briger *et al.*, 1958; Timothy *et al.*, 1961, 1963; Grobman *et al.*, 1961; Grant *et al.*, 1963; Brandolini, 1968; Mochizuki, 1968; Costa-Rodriguez, 1971; Paterniani and Goodman, 1977; Wellhausen, 1988; Avila and Brandolini 1990). Latin American countries, specifically Mexico, possess a great wealth of maize genetic diversity. There have been more than 40 land races of maize in Mexico (Wellhausen *et al.*, 1952; Hernández-Xolocotzi and Alanís, 1970; Ortega-Pazcka, 1980; Benz, 1986; Sánchez-González, 1989), and almost 250 land races in the Americas (Goodman and Brown, 1988).

#### C. Identification among *Zea mays* and wild species

The closest known relative of *Zea* is *Tripsacum*. The genus *Tripsacum* comprises two sections: section FASCICULATA with five species; and section TRIPSACUM with twelve species. The chromosome number varies from  $2n=36$  to  $2n=108$ . All species are perennials (deWet *et al.*, 1982, 1983). Twelve of these are native to Mexico and Guatemala with an extension of *T. dactyloides* throughout the eastern half of the United States, the tetraploids being near the East coast and the diploid in the central region. *T. lanceolatum* occurs in the southwest of the United States and *T. floridanum* is native to South Florida and Cuba. Three species of *Tripsacum* are known in South America.

Species of the section FASCICULATA are mostly and widely distributed in Meso-America, however, *T. lanceolatum* is found along the North of Sierra Madre Occidental, Mexico, up to Arizona. On the other hand, species of the section TRIPSACUM are distributed more extensively than the section FASCICULATA, although different species are found in relatively restricted territories; for example, *T. dactyloides* is found from a latitude about 42° North and 24° South. *T. dactyloides* tetraploid forms are also found in Kansas and Illinois in the United States. *T. manisuroides* is known only from Tuxtla Gutierrez, Chiapas, Mexico (deWet *et al.*, 1981, 1982, 1983). *T. andersonii* is of uncertain origin and is mostly sterile, it is an unusual species in that there is cytological (deWet *et al.*, 1983) and molecular evidence showing that its  $2n=64$  chromosomes comprise 54 *Tripsacum* chromosomes and ten *Zea* chromosomes (Talbert *et al.*, 1990).

#### D. Genetics and molecular identification

Maize has been one of the best studied plants in disciplines ranging from classical genetics to molecular biology. The study of maize has contributed to major breakthroughs in science such as the discovery of transposable elements (McClintock, 1929, 1934, 1944a, 1944b, 1944c, 1945; Fedoroff and Botstein, 1992). McClintock first characterised the ten chromosomes of maize using mitotic studies. Presently cytological research is being conducted on chromosome staining techniques, meiotic mutants, examination of the B chromosomes and better understanding of the events involved during synapsis. Transposable elements are very important in maize genetics. Many different transposable element systems have been described for maize, the best characterised has been the Activator (Ac) and Dissociation (Ds)

system. Ac/Ds comprises a family of maize transposable elements. Ac is the autonomous member of the family, capable of producing a transposable factor needed for mobility. Ds elements are not autonomous and capable of transposition only when trans-activated by Ac. Both genes have now been cloned and their mode of action is well characterised (Tsaftaris, 1995). A recent review of transposable elements is found in Federoff (2000).

The genetics of mitochondria and chloroplast in maize are of special importance. The mitochondrial genomes (mtDNAs) of higher plants are larger than those of mammalian or fungal mitochondrial genomes. The higher plant mitochondrial genomes are also more variable in their organization and have a larger coding capacity than mitochondrial genomes in mammals and fungi. Five types of mitochondrial genomes have been identified. Their designations are NA and Nb for the normal male fertile phenotypes, and T, S and C for the three different cytoplasmic male sterile (cms) phenotypes. Physical maps for three of the maize cytotypes have been completed. Mitochondrial genomes of higher plants have integrated DNA sequences that originate from other cell compartments (Tsaftaris, 1995). In contrast to plant mitochondria genomes, the chloroplastic genome is smaller and simpler; thus many chloroplastic genomes have been completely sequenced. The similarities between the genomes of chloroplasts and bacteria are striking. The basic regulatory sequences, such as transcription promoters and terminators, are virtually identical in both cases. Protein sequences encoded in chloroplasts are clearly recognisable as bacterial, and several clusters of genes with related functions are organised in the same way in the genomes of chloroplasts, *E. coli*, and cyanobacteria. In about two-thirds of higher plants, including maize, the chloroplast as well as mitochondrial DNA, is maternally inherited (Tsaftaris, 1995).

There is an abundant literature on the genetics, physiology, cytogenetics and molecular biology of maize and concise, thorough reviews are available (Coe *et al.*, 1988; Carlson, 1988; Walbot and Messing, 1988; Hageman and Lambert, 1988; Freeling and Walbot, 1994).

#### E. Maize genome maps

The first RFLP map of corn was developed by Helentjaris *et al.*, (1985, 1986a, 1986b). The corn linkage map encompasses approximately 1200 map units. The RFLP markers are not randomly distributed. The corn genome is about  $5 \times 10^6$  kb, then there would be approximately  $4 \times 10^3$  kb per map unit. It includes highly repeated sequences that constitute about 20% of the genome; these sequences are present in about ten superabundant sequence types. There are more than 1000 different moderately repetitive sequence families collectively representing 40% of the genome, this leaves approximately 40% single copy sequences, or more than  $10^6$  approximately gene size pieces.

Maize has one of the most well saturated genetic maps of any cultivated plant of this genome size. In principle this offers the possibility of easily locating any transgene and/or identifying any specific genotype (Tsaftaris, 1995). Recent maize genome maps and most of the information on the maize genome can be found in the following web addresses: <http://www.agron.missouri.edu>; <http://www.zmdb.iastate.edu>; <http://w3.aces.uiuc.edu/maize-coop/>. An expressed sequence tag (EST) database can also be found at <http://www.zmdb.iastate.edu>.

#### 4. Centre of Origin /Diversity, Maize Diversity

There are four main hypotheses on the origin of maize.

- **The descent from teosinte hypothesis.** This is the oldest proposal and was advanced by Ascherson in 1895 (Mangelsdorf and Reeves, 1939) and proposes that maize was domesticated from teosinte by human selection. This is the most widely accepted hypothesis at present (Beadle, 1986; deWet and Harlan, 1972; Doebley and Stec, 1991; Doebley, 1990; Galinat, 1977; Iltis and

Doebley, 1980; Goodman, 1988; Kato, 1984; Kato and López, 1990; Timothy *et al.*, 1979). The main problem with this hypothesis was how the distichous small female spike could have been transformed into the polistichous gigantic maize spike (ear) by human selective domestication. However, Doebley *et al.*, (1990) have found five major genes controlling 'key' traits distinguishing maize and teosinte, and more recently Wang *et al.*, (1999) have discussed a gene controlling the inflorescence character in teosinte and maize.

- **The tripartite hypothesis.** The main assumption of this hypothesis is that there existed a wild maize in the past, which is considered extinct at present. This wild maize gave origin to the annual teosintes by crossing with *Tripsacum*. Further crossing of teosinte with wild maize gave rise to the modern races of maize (Mangelsdorf and Reeves, 1939; and Mangelsdorf, 1974). Later on Mangelsdorf *et al.*, (1981) based on experimental crossing between *Z. diploperennis* and the race Palomero Toluqueño of maize and further observations of its progenies, proposed that the annual teosintes are the products of this crossing. The fact that until now no evidence at all has been found about the existence, in the past or at present, of a wild maize, this hypothesis has lost much credence with time (although see Eubanks, 1995).
- **The common origin hypothesis.** This hypothesis proposes that maize, teosinte and *Tripsacum* originated by "ordinary divergent evolution" from a common ancestor. Consequently, it is conceived that there existed a wild maize plant that further was transformed into a cultivated plant by the selection and care of man (Weatherwax, 1955; Randolph, 1955; Randolph, 1959). The postulation that wild maize existed in the past makes this hypothesis not acceptable, as in the case of the tripartite hypothesis.
- **The catastrophic sexual transmutation hypothesis.** This hypothesis proposes that the maize ear evolved from the terminal male inflorescence of teosinte lateral branch by a "... sudden epigenetic sexual transmutation involving condensation of primary branches [and further] genetic assimilation under human selection of an abnormality, perhaps environmentally triggered" (Iltis, 1983). The finding of five mutant genes controlling key characters separating maize from teosinte (Doebley and Stec, 1991; Doebley *et al.*, 1990) seems to make the catastrophic sexual transmutation hypothesis untenable.

### *Centre of maize domestication*

The Meso-American region located within middle South Mexico and Central America is recognised as one of the main centres of origin and development of agriculture as well as centre of origin and diversification of more than one hundred crops (Vavilov, 1951; Smith, 1995; Harlan, 1992). At the present time, there is no agreement about where exactly maize was domesticated and there are several proposals in this regard. Based on the findings of archaeological materials from the maize plant (pollen, cobs, husks, and other remnants) in the United States and Mexico, which are older than those found in South America, Randolph (1959) proposed that maize was domesticated, independently, in the southwestern United States, Mexico, and Central America.

Mangelsdorf (1974) proposed that "corn had not one origin but several in both Mexico and South America", because the archaeological evidences are found in Mexico and several morphological characteristics in extant population found in the maize races of South America (Andes region) in comparison to those races of Meso-America.

The preliminary studies of McClintock (1959, 1960) on the chromosome knob constitutions of several races of maize from South America, Mexico and Central America, led her to conclude "that present-day maize may have derived from several different centres". These chromosome studies were further exploited

(Kato, 1976, 1984; McClintock, 1978; McClintock *et al.*, 1981). They confirmed McClintock's previous conclusion and led to the proposal that maize was domesticated, independently, in four centres located in Mexico (two in Oaxaca-Chiapas region, one in the central highlands and one in the mid-highlands of Morelos-northern Guerrero), and one in the highlands of Guatemala. "This conclusion is based on the fact that chromosome knobs are not geographically and racially distributed at random, and that some knobs show restricted distributions following clear-cut pathways through specific territories, dispersion that clearly indicate that they were started in specific regions or centres of distribution. These centres are then considered as the places where original maize germplasm was domesticated from teosinte populations that were already cytogenetically well diversified" (Kato, 1984).

Contrary to the above multicentres origin of maize proposals, the isoenzymatic variation studies of maize and teosinte suggested to Doebley *et al.*, (1987a) that maize was domesticated once in the Balsas basin region because "... all maize races of Mexico are isoenzymatically closer to *var. parviglumis* than to other teosintes...". Supporting this hypothesis, further molecular genotyping studies also suggest that maize originated from a single domestication in southern Mexico 9000 years ago (Matsuoka *et al.*, 2002).

### *Maize diversity*

From the time of the discovery of America, Columbus noted the presence of corn on the North coast of Cuba and introduced it to Europe through Spain. At that time, corn was grown from Chile to southeastern Canada. Within two generations, after its introduction in Europe, corn became a cultivated crop throughout the world (Goodman, 1988). Germplasm resources are preserved *ex-situ* in many parts of the world, however, only in the Meso-American region there still exists, *in situ*, the original ancient maize that gave rise to improved varieties that are grown in all regions of the world. Most of the maize variation can be found in the Meso-American region and the northern part of South America. The great diversity of environments and conditions have created the basis for the development of maize varieties well adapted to harsh conditions of soil and climate as well as to biotic stresses. There is a close correlation among community culture, production system and the type of consumption of maize, with the diversification and variation of maize (Aguirre *et al.*, 1998; Louette and Smale, 1998).

Maize germplasm diversity is threatened by several factors: improved seed adoption; shift to cultivation of cash crops; and change in land use (Aguirre *et al.*, 1998; Bellon *et al.*, 2000; Louette, 1997). In some areas the adoption of hybrids and improved seed has increased dramatically, which has reduced the production of maize for traditional uses and, consequently, the increase of genetic erosion. Although these factors play an important role in reducing maize germplasm diversity, the persistence of maize land races in the Central American region is evident. Small farmers, peasants and indigenous ethnic groups and communities in many Latin American countries still preserve and select traditional maize.

Some arguments to explain the maize land race survival have been advanced (Ortega-Pazcka, 1973). The paramount importance of native maize for small communities, ethnic groups, small farmers and peasants, resides in the fact that land races of maize have very specific qualities for food and special uses as mentioned in Section 1, rather than maize yield itself; therefore, many land races of maize have not been displaced by more productive maize types promoted by governmental agencies. For example, in Mexico after 50 years of maize genetic improvement programs, the adoption of hybrids and improved varieties is low. The research of Hernández-Xolocotzi (1972), Ortega-Pazcka (1973), Benz (1986), and Ortega-Pazcka *et al.*, (1988), on maize diversity and peasant communities, demonstrates that local maize has been preserved by peasants, using traditional methods, basically intact for decades. As the result of a poll carried out in 1992 (CIMMYT, 1994), it was concluded that open pollinated land races of maize cover 42% of arable land dedicated to maize in less developed countries.

The approach for conservation of Latin American maize land races relies on two main criteria: the adaptation to a particular ecological niches and special forms of consumption of specific land races. Native germplasm utilisation has varied depending on the country and the needs of development. In general, the strategy is to identify sources of elite germplasm by means of characterizing and evaluating samples from land race collections, consisting of composite groups, populations and pools. National programs, international institutions, private seed industries and universities use these germplasm materials. Native maize land races have not been widely used for improvement programs and in Mexico, for example, only 10% of Mexican maize land races have been incorporated in specific breeding programs. There are a couple of examples in Mexico where native races of maize were characterised and evaluated for selection to generate improved populations, which were released as new open pollinated varieties: variety V520 (from land race San Luis Potosí-20); and variety Rocamex V7 (from land race Hidalgo-7). However, there is still germplasm in farmers' fields that have not been evaluated for their improvement and utilisation (Márquez-Sánchez, 1993).

Examples of maize land races specifically adapted to special conditions are (Hernández-Xolocotzi, 1988): Gasepe, short growing season (early maturity); Guatemalan Big Butt, long growing season (late maturity); Tuxpeño, Celaya, Chalqueño, Cuban Yellow Flint and Cuzco Gigante, high efficiency and productivity under good rainfed conditions; Chococeño, Enano and Piricincó, tolerance to high temperature and humidity; Cónico norteño, tolerance to semi-dry environments; Palomero Toluqueño, Cónico, Cacahuacintle and Sabanero, well adapted to high elevations, low temperature; Nal-tel, adapted to calcareous soil.

## 5. Reproductive Biology

### A. Sexual reproduction

*Zea mays* is an allogamous plant that propagates through seed produced predominantly by cross-pollination and depends mainly on wind borne cross-fertilisation. *Z. mays* is a plant with a protandrous inflorescence; however, decades of conventional selection and improvement have produced varieties of maize with protogynous traits. *Z. mays* has staminate flowers in the tassels and pistillate flowers on the ear shoots.

The tassel. The structure and development of the stamens are similar to other grasses. The anther develops four chambers or loculi each one containing a central row of archeosporial cells that gives rise to sporogenous tissue. After seven weeks the microspore mother cells are in the meiosis stage. Microspores are organised around four nuclei and become mature pollen grains. The amount of pollen produced by a tassel is estimated at 18 million pollen grains (Kiesselbach, 1980). Probably the best-improved varieties would produce more than this. On average 21,000 pollen grains could be produced for each kernel on an ordinary ear with 1000 kernels. Kiesselbach (1980) calculated that: "With a stand of three stalks in hills 42 inches apart, an area of 588 square inches is available in the field for each stalk. Thus an average of 42,500 pollen grains are provided for each square inch of the field. If the silks of an ear display a total surface of 4 square inches they will intercept about 170,000 pollen grains. Estimating 1,000 silks per ear, this amounts to 170 pollen grains per silk. Considering that corn in the field sheds pollen for 13 days, each silk receives an average of 13 pollen grains per day."

The ear shoot. At each node of the stem there is an axillary bud enclosed in the prophyllum. Only one or two of these axillary buds will develop as ear shoot and reach the fertilisation stage. At first the ear is smooth but protuberances soon form in rows. The basal protuberances are formed first and development advances towards the tip of the ears. Each one becomes two lobed, each lobe developing into a spikelet with two flowers, only one of which commonly persists. The growing point of the upper flower is differentiated to form the functional pistil. The part above the attachment of the carpels develops a single

sessile ovule, which consists of a nucellus with two integuments or rudimentary seed coats. The united carpels, which will form the ovary wall or pericarp of the mature kernel, grow upward until they completely enclose the ovule. Where they meet, the functionless so-called stylar canal is formed. The two anterior carpels, which face the ear tip, form outgrowths, which develop into the style or silk. The surface of the silk becomes covered with numerous hairs, which are developed from cells of the epidermis. At the base of the silk is a growth zone where new cells develop, causing continuous elongation of the silk until it is pollinated and fertilisation takes place. The development of the embryo sac is characteristic of the grass family. One of the three nuclei at the micropylar end enlarges and becomes the nucleus of the egg, while the others become the nuclei of the synergids. At this stage the embryo sac is ready for fertilisation but if pollination is prevented it may remain in this condition for some time, perhaps two weeks, after which the embryo sac and nucellus disorganise and fertilisation is no longer possible.

Fertilisation occurs after the pollen grain is caught by the silk and germinates to create the pollen tube which penetrates up to the micropyle and enters the embryo sac. The pollen is carried mainly by wind, thus it is highlighted that pollination can occur even, although rarely, over long distances measured in kilometers.

## B. Asexual reproduction

There is no asexually reproductive maize. Cell/tissue culture techniques can be used to propagate calli and reproduce tissues or plants asexually; however, with maize cells and tissues these techniques are difficult.

## 6. Crosses

### A. Intra-specific crosses

Maize is essentially 100% open-pollinated (cross-fertilising) crop species. Until the 20<sup>th</sup> century, corn evolved through open pollinated varieties, which are a collection of heterozygous and heterogeneous individuals developed by mass selection of the people from the different civilisations existing in the Americas (Hallauer, 2000). Corn pollen is very promiscuous, lands on any silk, germinates almost immediately after pollination, and within 24 h completes fertilisation. Thus all corns will interpollinate, except for certain popcorn varieties and hybrids that have one of the gametophyte factors of the allelic series *Ga* and *ga* on chromosome four (Kermicle, 1997).

There is a great sexual compatibility between maize and annual teosinte and it is known that they produce fertile hybrids (Wilkes, 1977). In areas of Mexico and Guatemala maize and teosinte freely hybridise when in proximity of each other. Wilkes (1977) reported a frequency of one F1 hybrid (corn x teosinte) for every 500 corn plants or 3 to 5 % of the teosinte population for the Chalco region of the Valley of Mexico. Kermicle and Allen (1990) have shown that maize can introgress to teosinte; however, there is incompatibility between some maize populations and certain types of teosinte resulting in low fitness of some hybrids that prevents a high rate of introgression (Evans and Kermicle, 2001).

### B. Inter-specific crosses

Although it is extremely difficult, *Tripsacum* species (*T. dactyloides*, *T. floridanum*, *T. lanceolatum*, and *T. pilosum*) can be crossed with corn; however, hybrids have a high degree of sterility and are genetically unstable (Mangelsdorf, 1974). Galinat (1988) advanced that since *Tripsacum* and *Zea* have different chromosome numbers, the addition of an extra *Tripsacum* chromosome into the maize genome would occur with a low frequency and consequently the rate of crossing-over would be extremely reduced. Despite these arguments, Eubanks (1995, 1998) developed a method for transferring *Tripsacum* genes into maize. In this method two wild relatives of maize, *Tripsacum* and diploid perennial teosinte (*Zea*

*diploperennis*), are crossed to produce a hybrid, which is called tripsacorn, used to generate maize-tripsacorn hybrids. The use of tripsacorn is intended to confer resistance to pests and disease, drought tolerance and improved uniformity. Recently it has been claimed (Eubanks, 2000) that traits such as apomixis, totipotency, perennialism, adaptation to adverse soil conditions and to carbon dioxide enriched atmosphere can be transmitted to maize via maize x *Tripsacum*-perennial teosinte (and/or its reciprocal).

The cross between maize and *Tripsacum* has been studied since long ago (deWet *et al.*, 1973; Bernard and Jewell, 1985), and recently efforts have been made to transfer genes related to traits like apomixis from *Tripsacum* to maize (Burson *et al.*, 1990; Savidan and Berthaud, 1994; Hanna, 1995; Leblanc *et al.*, 1995; Grimanelli *et al.*, 1998; Grossniklaus *et al.*, 1998). Maize x *Tripsacum* hybrids have been produced and consequently several patents on apomictic maize have been published (Kindiger and Sokolov, 1998; Savidan *et al.*, 1998; Eubanks, 2000).

### C. Gene flow

The interaction between domesticated plants and their wild relatives can lead to hybridisation and in many cases to gene flow of new alleles from a novel crop into the wild population (Ellstrand *et al.*, 1999). While gene flow *per se* is not a concern, theoretically, it can lead to the potential for the evolution of aggressive weeds or the extinction of rare species. There has been preliminary documentation of this in some cases although not for maize (Ellstrand *et al.*, 1999).

As mentioned in Section VI-A some teosinte species can produce fertile hybrids with maize. All teosintes, members of the Section LUXURIANTES and subspecies *mexicana* and *parviglumis*, occur only in Mexico and Guatemala (Sánchez-González and Ruiz-Corral, 1997). It has been documented that maize and teosinte often interact, particularly with *Zea mays* ssp. *mexicana* (Wilkes, 1977). Also, the known distribution of teosintes, together with high likelihood of the presence of land races in the maize production areas of Mexico indicates, as shown in Appendix II, that there exist high probabilities of genetic exchange between conventional maize, land races and teosinte (Sánchez-González and Ruiz-Corral, 1997; Serratos-Hernández *et al.*, 1997; Serratos-Hernández *et al.*, 2001). However, there is some evidence of restricted gene flow between *Zea* spp. that occurs predominantly from teosinte into maize (Doebley *et al.*, 1987a). To date, there is no genetic analysis of morphologically intermediate plants that could identify “whether the maize-teosinte intermediates are true hybrids, introgressants or crop mimics” (Ellstrand *et al.*, 1999). Out-crossing of maize with *Tripsacum* species is not known to occur in the wild.

Another factor to take into account regarding gene flow is the exchange of seed and traditional maize improvement practised by peasant communities and small farmers. As observed by Louette (1997), rural communities are open systems where “...there is a constant flow of genetic material among communities over large areas.” therefore, as in the case of Mexico, “...a land race variety, an improved variety, or a transgenic variety of maize, can reach any zone of the country, even the most isolated ones, such as those where teosinte grows.” The human factor together with the changes in policy and strategies in maize production (Nadal, 1999) may increase several fold the chance of gene flow between improved maize, teosinte and landraces.

## 7. Agro-ecology

### A. Cultivation

Although maize was domesticated and diversified mostly in the Meso-American region, at present it is cultivated mainly in warm temperate regions where the conditions are best suited for this crop (Norman *et al.*, 1995).

Maize is an annual plant and the duration of the life cycle depends on the variety and on the environments in which the variety is grown (Hanway, 1966). Maize cannot survive temperatures below 0° C for more than 6 to 8 hours after the growing point is above ground (5 to 7 leaf stage); damage from freezing temperatures, however, depends on the extent of temperatures below 0° C, soil condition, residue, length of freezing temperatures, wind movement, relative humidity, and stage of plant development. Light frosts in the late spring in temperate areas can cause leaf burning, but the extent of the injury usually is not great enough to cause permanent damage, although the corn crop will have a ragged appearance because the leaf areas damaged by frost persist until maturity. Maize is typically grown in temperate regions due to the moisture level and number of frost-free days required to reach maturity. The number of frost-free days dictates the latitude at which corn varieties with different life cycle lengths can be grown. Maize having a relative maturity of 100 to 115 days is typically grown in the U.S. corn belt. Maize varieties with different relative maturities do not occur in parallel east-to-west zones because they are also dependent on prevailing weather patterns, topography, large bodies of water, and soil types (Troyer, 1994 in Hallauer, 2000).

In tropical regions, maize maturity increases due to altitude effects. Tropical land races of maize in the tropics characteristically show three to five ears and axillary tillering, as opposed to modern cultivars that suppress lower ears and tillers (Norman *et al.*, 1995). In the tropics Oxisols, Ultisols, Alfisols and Inceptisols are best suited for maize production; however, maize is adapted to a wide variety of soils in the tropics, from sands to heavy clay. Of particular importance is aluminium toxicity for maize on acid tropical soils. Liming can solve this problem, "Deep lime incorporation in the subsoil of some Oxisols has overcome aluminium toxicity, thereby improving rooting depth in maize and tolerance to dry periods" (Norman *et al.*, 1995).

The farmland of Mexico covers a wide range of ecological conditions: from sea level to 2800 meters, from very dry to wet climates, well drained to poorly drained soils, flat to severe slopes, shallow to deep soils, low to high solar radiation; drought, wind and frost damage are common.

The poorest farmers are typically Indian farmers that inhabit the Sierras. Dry beans, squash, grain amaranth and several other species were also domesticated by the inhabitants of the region, as complements to their diet. They also developed the typical "milpa cropping system" as a cultivated field that may involve the association, inter-cropping, or relay-cropping of maize, beans, squash, grain amaranth, tree species and several tolerated herbal species. The isolation of these farming communities has caused the development of a great resource of maize germplasm diversity, which is conserved using *in situ* and *ex situ* (germplasm banks) means. Inter-cropping of maize with other crops is practiced in many areas of less developed countries (Norman *et al.*, 1995). These systems imply changes at the level of cultivation and management of maize production which are important in terms of ecological relationships.

## **B. Volunteers and weediness**

Maize has lost the ability to survive in the wild due to its long process of domestication, and needs human intervention to disseminate its seed. Although corn from the previous crop year can over-winter and germinate the following year, it cannot persist as a weed. The presence of corn in soybean fields following the corn crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with the hoe or use of herbicides to kill the plants in soybean fields, but the plants that remain and produce seed usually do not persist during the following years. Volunteers are common in many agronomic systems, but they are easily controlled; however, maize is incapable of sustained reproduction outside of domestic cultivation. Maize plants are non-invasive in natural habitats (Gould, 1968). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Consequently seed dispersal of individual kernels does not occur naturally. Individual kernels of corn, however, are distributed in fields and main avenues of travel from the field operations of harvesting the crop and transporting the grain from the harvested fields to storage facilities (Hallauer, 2000).

### **C. Soil ecology (Microbiology of maize rhizosphere)**

Maize root system acts as a soil modifier due to its association with several microbial groups such as bacteria, fungi, actinomycetes (Vega-Segovia and Ferrera-Cerrato, 1996a), protozoa and mites. The highest microbial population usually is bacteria, followed by fungi and actinomycetes. All these microbial groups play a particular role in the soil ecology, such as nutrimental cycling and the availability of nutrients for plant growth. In addition, these microbial organisms contribute to the protection of the root system against soil pathogens.

Some research has been oriented to understand more on microbial activity and its physiology. For instance, the physiology of free nitrogen-fixing bacteria such as *Azotobacter*, *Beijerinckia* and *Azospirillum* which have been found in the rhizosphere of several maize cultivars and teosinte (González-Chávez *et al.*, 1990; González-Chávez and Ferrera-Cerrato, 1995; Vega-Segovia and Ferrera-Cerrato, 1996b).

There is information related to symbiosis with arbuscular mycorrhizal fungi (AMF) which shows that these endophytes associate with specific maize genotypes (González-Chávez, and Ferrera-Cerrato, 1989; González-Chávez and Ferrera-Cerrato, 1996). There are reports related to the capability of a single AMF to establish symbiosis with a wide range of maize land races and teosinte (Santamaría and Ferrera-Cerrato, 1996; Benítez *et al.*, unpublished data). All these materials are used in Mexican agriculture. The role of these symbiosis relationships is to increase root metabolism in order to improve phosphorus uptake.

A great deal of life diversity is associated with maize grown in the milpa system of the Sierras. One example is the adaptation developed by a type of maize race in the Mixe Sierra of Oaxaca. The brace roots are overdeveloped and covered by a mucilaginous material that harbours species of nitrogen fixing free bacteria (R. Ferrera-Cerrato, personal comm.).

Soil ecology studies are undertaken to identify micro-organisms with agricultural value in places where maize is cultivated (Pérez-Moreno and Ferrera-Cerrato, 1997). Nowadays, these micro-organisms are being studied for the potential to augment corn cultivation. Selective breeding and nutrient management are also being evaluated for enhancing maize production.

### **D. Maize-insect interactions**

In Appendix III, a list of common insect pests and pathogens of maize is presented.

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## APPENDIX I

### Maize Biotechnology

For practical purposes maize biotechnology could be divided into two fields: genetic engineering and molecular genetics.

Molecular genetics refers to the identification and location (genome mapping) of genes within the genome of organisms by means of molecular techniques that make use of the chemical properties of DNA (Hoisington *et al.*, 1998). The marker technologies presently available for genomics work are: 1) Restriction Fragment Length Polymorphisms (RFLPs); 2) Random Amplified Polymorphic DNAs (RAPDs); 3) Sequence Tagged Sites (STSs); 4) Simple Sequence Repeats (SSRs); 5) Amplified Fragment Length Polymorphisms (AFLPs); and 6) Single Nucleotide Polymorphisms (SNPs). These technologies have been applied in maize breeding through fingerprinting for identification of genotypes, monitoring genetic diversity and for the efficient management of genetic resources (Hoisington *et al.*, 1998). Other applications of molecular genetics and molecular markers are 1) Comparative Mapping, and 2) Marker Assisted Selection.

Genetic engineering methodologies can make possible the insertion of foreign DNA, from organisms of different species, into another individual organism. In maize, at the commercial level, the introduction of foreign DNA has been successfully accomplished through a technique known as biolistics. In this technique, DNA coated microparticles are shot by means of an air compression device, to cells in plant tissue or callus. In the case of maize, embryogenic callus is used for bombardment with foreign DNA. To identify the cells that have taken up the foreign DNA in maize, a herbicide resistant selectable gene has been used. Fertile transgenic maize plants have also been produced using 1) PEG-mediated protoplast transformation; 2) electroporation of intact or partly degraded cells of immature embryos, callus or embryonic suspensions; 3) 'whiskers' technology; and 4) *Agrobacterium*-mediated transformation.

At present there are two types of commercially released transgenic maize produced by means of genetic engineering: 1) Insect pest resistant maize or Bt-maize; and 2) Herbicide resistant maize. However, more research and development in this area is underway. Transgenic maize with elevated (10 KD) zein and methionine has been obtained (Anthony *et al.*, 1997). Antifungal proteins, such as chitinases and beta-1,3-glucanases, have been genetically engineered to attempt expression in the maize kernels with the aim to prevent the growth of *Aspergillus flavus* and the production of aflatoxins (Duncan *et al.*, 1985; Wu *et al.*, 1994; Wan *et al.*, 1995). Transgenic maize will serve as bioreactors for producing various biomolecules with applications in food, feed and the pharmaceutical industry (Nikolov, 1999).

The complicated and plastic nature of organellar genomes especially those of maize mitochondria, requires special consideration for the stability of the cytoplasmic male sterility genes if they are used for preventing pollen formation. Equally these features of organelle genomes would also apply to any genes cloned into them (since recent developments indicate that organelles could be a better target for generating transgenic plants). Therefore, stable incorporation of a transgene into the plastid genome guarantees amplification of the transgene, potentially resulting in a very high level of foreign gene expression. Since chloroplast (and mitochondrial) genomes resemble the genomes of other organisms and are most probably evolutionarily related, the possible transfer of genes from these organelles to microorganisms should be studied in the future if more and more transgenes are targeted to these organelles

The great similarity between the chloroplastic genome and microbial genomes was one of the reasons for choosing the chloroplast as a target for transferring native microbial genes to plants. For instance since the transcriptional machinery of the plastid is prokaryotic in origin and its genome is relatively A-T rich, it was possible that native Bt toxin genes from *B. thuringiensis* might be efficiently expressed in this organelle without nuclear modification. In addition, plant cells may contain up to 50,000 copies of the circular plastid genome.

Transposable elements are not expected to affect transgenes differently from their reported effects on non-modified genes of maize, unless sequences of the transposable element are contained in the inserted genetic material (Tsaftaris, 1995).

The potential crossing of landrace maize germplasm with transgenic improved maize, hybrids or inbreds should be considered carefully since, for example in Mexico, it is well known the high incidence of transposable elements in landraces of maize (Gutiérrez-Nava *et al.*, 1998).

Several investigations conducted by national and international research institutions have demonstrated that gene exchange between improved maize and landraces is a continuing process taking place in small farmers' corn fields. The report on the presence of transgenes in peasants' maize fields of Oaxaca (Quist and Chapela, 2001), have been further demonstrated by the Mexican government (INE-CONABIO, 2001), confirming that gene movement in traditional agriculture is an open system.

### *Weediness of transformed corn varieties*

Gene transformation is the acquisition by a cell of new gene(s) by the uptake of naked DNA, which in the case of maize can be by direct introduction of DNA. As stated before, the more common applications of gene transfer in corn are insect resistance or tolerance to herbicides. Herbicide tolerance is usually conferred by single genes that interact with key enzymes in important metabolic pathways. Insect resistance is conferred by the expression of an insecticidal protein from *B. thuringiensis*. The overall phenotype of transformed plants with these two types of genes is similar to the original phenotype: the reproductive organs (tassels and ears), duration of plant development, methods of propagation, ability to survive as a weed, will not change with these two types of genes.

Gene exchange between cultivated corn and transformed corn would be similar to that which naturally occurs at the present time. Wind-blown pollen would move about among plants within the same field and among plants in nearby fields. Free flow of genes would be similar to that which occurs in cultivated corn. The transformed plants include individual genes, and depending on the relative expression of the transformed genes (relative levels of dominance for gene expression), plant architecture and reproductive capacities of the inter-crossed plants will be similar to non-transformed corn. With the transgenic maize that is available at this moment in the world, the chance that a weedy type of corn will result from inter-crossing of transgenic maize with cultivated conventional maize is remote.

Out-crossing of transformed corn plants with wild relatives of corn will be the same as for non-transformed corn plants. Out-crossing with teosinte species will only occur where teosinte is present in Mexico, Guatemala and probably in some other places of Central America. Out-crossing with *Tripsacum* species is not known to occur in the wild.

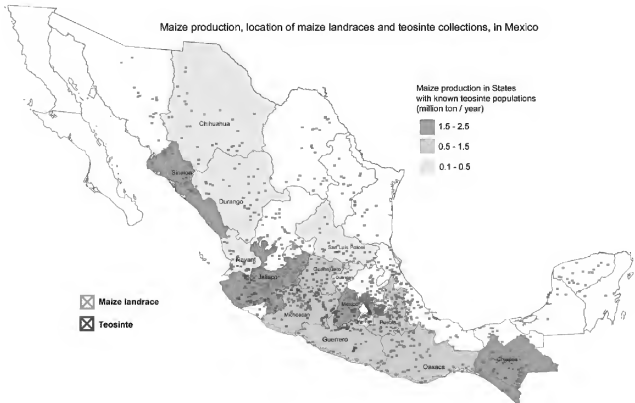
### *Unintended effects*

The commercial release of transgenic maize expressing delta-endotoxin from *Bacillus thuringiensis* has driven the interest of ecologists concerned with the evolution of pest resistance to pesticide plants (Bergvinson *et al.*, 1997; Willcox and Bergvinson, 1997; Marvier, 2001; Obycki *et al.*, 2001). The evolution of pest resistance is commonly known in any system where negative selection occurs from the

use of traditional chemical pesticides, including plants bred traditionally for pest resistance. Recently, an effect of pollen from transgenic maize on the monarch butterfly larvae, a non-target insect, has preliminarily been described (Losey *et al.*, 1999). However, recent studies in the field have shown a less dramatic effect on non-target organisms (Wraight *et al.*, 2000; Hellmich *et al.*, 2001; Sears *et al.*, 2001; Zangerl *et al.*, 2001).

## APPENDIX II

### Distribution of Maize Landraces and Teosinte in Mexico



Source: Serratos-Hernández *et al.*, 2001.

## APPENDIX III

## Common Diseases and Insect Pests of Maize (CIMMYT and DGSV Guides)

Maize	Insect pests	Diseases
Stalk	Termites ( <i>Coptotermes formosanus</i> ), Sugarcane borer ( <i>Diatraea saccharalis</i> ), Southwestern corn borer ( <i>Diatraea grandiosella</i> ), Neotropical corn borer ( <i>Diatraea lineolata</i> ), Asian maize borer ( <i>Ostrinia furnicalis</i> ), Spotted sorghum stem borer ( <i>Chilo partellus</i> ), African maize stem borer ( <i>Busseola fusca</i> ), African pink borer ( <i>Sesamia calamistis</i> ), African sugarcane borer ( <i>Eldona saccharina</i> ), Maize stem weevils ( <i>Cilindrocopturus adpersus</i> ), European corn borer ( <i>Ostrinia nubilalis</i> ).	Charcoal rot ( <i>Macrophomina phaseoli</i> ), Diplodia stalk rot ( <i>Diplodia maydis</i> ), Gibberella stalk rot and Fusarium stalk rot ( <i>Fusarium</i> spp.), Brown spot ( <i>Physoderma maydis</i> ), Black bundle disease ( <i>Cephalosporium acremonium</i> ), Late wilt ( <i>Cephalosporium maydis</i> ), Maize bushy stunt disease (MBSD), Botryodiplodia stalk rot ( <i>Botryodiplodia theobromae</i> ), Maize lethal necrosis (simultaneous infection of maize chlorotic mottle virus and either maize dwarf mosaic virus or wheat streak mosaic virus), Maize chlorotic mottle virus (MCMV), Corn stunt disease ( <i>Spiroplasma</i> ), Pythium stalk rot ( <i>Pythium aphanidermatum</i> , <i>Pythium</i> spp.), Erwinia stalk rot ( <i>Erwinia carotovora</i> f. sp. <i>zeae</i> )
Leaf	Corn stunt leafhoppers ( <i>Dalbulus maidis</i> ), Maize streak virus leafhoppers ( <i>Dalbulus maidis</i> , <i>D. elimatus</i> ), Fall armyworm ( <i>Spodoptera frugiperda</i> ), Armyworm ( <i>Mythimna unipuncta</i> ), Spider mites ( <i>Oligonychus mexicanus</i> ), Corn leaf aphid ( <i>Rhopalosiphum maidis</i> , <i>R. padi</i> ), Maize Whorl Maggots ( <i>Euxesta</i> spp.), Sugarcane Froghoppers ( <i>Aeneolamia postica</i> , <i>Prosapia simulans</i> ), Chafers, Grasshoppers ( <i>Sphenarium</i> spp., <i>Melanoplus</i> spp.).	Downy mildew ( <i>Sclerospora</i> spp., <i>Sclerophthora</i> spp), Curvularia leaf spot ( <i>Curvularia lunata</i> and <i>Curvularia pallescens</i> ), Cercospora leaf spot ( <i>Cercospora zeae-maydis</i> ), Septoria leaf blotch ( <i>Septoria maydis</i> ), Turicum leaf blight ( <i>Helminthosporium turcicum</i> ), Diplodia macrospora leaf stripe ( <i>Diplodia macrospora</i> ), Phyllosticta leaf spot ( <i>Phyllosticta maydis</i> ), Helminthosporium carbonum leaf spot ( <i>Helminthosporium carbonum</i> ), Bacterial leaf stripe ( <i>Pseudomonas rubriligneas</i> ), Eyespot of maize ( <i>Kabatiella zeae</i> ), Leptosphaeria leaf spot ( <i>Leptosphaeria michotii</i> ), Maydis leaf blight ( <i>Helminthosporium maydis</i> ), Stewart's wilt ( <i>Erwinia stewartii</i> ), Maize dwarf mosaic (MDMV), Southern rust ( <i>Puccinia polysora</i> ), Common rust ( <i>Puccinia sorghi</i> ), Tropical rust ( <i>Physopella zeae</i> ), Zonate leaf spot ( <i>Gloeocercospora sorghi</i> ), Banded leaf and sheath spot ( <i>Rhizoctonia solani</i> f. sp. <i>sasakii</i> ), Tar spot ( <i>Phyllachora maydis</i> ), Brown spot ( <i>Physoderma maydis</i> ) leaf anthracnose ( <i>Colletotrichum graminicola</i> ), Phaeosphaeria leaf spot, Fine stripe virus, Corn streak virus, Bacterial leaf stripe, Maize chlorotic mottle virus, Fine stripe virus, Fine mosaic virus I, Corn stunt disease, Black bundle disease.
Ear	Ear maggots, Corn earworms ( <i>Helicoverpa zea</i> ), Stink bugs ( <i>Euschistus servus</i> , <i>Nezara viridula</i> ), Angoumois grain moth ( <i>Sitotroga cerealella</i> ), Indian meal moth ( <i>Plodia interpunctella</i> ), Grain weevils ( <i>Sitophilus granarius</i> , <i>S. zeamais</i> ), Grain borers ( <i>Prostephanus truncatus</i> ).	Corn stunt disease, Botryodiplodia, Penicillium ear rot, Cladosporium ear rot, Gibberella ear rot, Maydis leaf blight (T strain), Nigrospora ear rot, Tar spot, Black bundle disease, Maize dwarf mosaic, Downy mildew, Gibberella ear rot, Helminthosporium carbonum ear rot, Banded leaf and sheath spot, Ergot of maize, Head smut, Aspergillus ear rots, Banded leaf and sheath spot, Maize stripe virus, Comon smut, Gray ear rot, Diploidia ear rot, Charcoal ear rot.

Tassel	Corn stunt leafhoppers ( <i>Dalbulus maidis</i> ), Maize streak virus leafhoppers ( <i>Dalbulus maidis</i> , <i>D. elimatus</i> ), Fall armyworm ( <i>Spodoptera frugiperda</i> ), Armyworm ( <i>Mythimna unipuncta</i> ), Spider mites ( <i>Oligonychus mexicanus</i> ), Corn leaf aphid ( <i>Rhopalosiphum maidis</i> , <i>R. padi</i> ), Maize Whorl Maggots, Sugarcane Froghoppers ( <i>Aeneolamia postica</i> , <i>Prosapia simulans</i> ), Chafers, Grasshoppers ( <i>Sphenarium</i> spp., <i>Melanoplus</i> spp.).	Head smut, Downy mildew, Maize chlorotic mottle virus, Bacterial leaf stripe, False head smut, Corn stunt disease, Maize stripe virus.
Seed, Root, and Seedling	Seedcorn maggots ( <i>Hylemya platura</i> ), Wireworms ( <i>Agriotes lineatus</i> ), Flea beetles ( <i>Phyllotreta</i> spp.), Diabrotica beetles ( <i>Diabrotica</i> spp.), Maize billbugs ( <i>Sphenophorus maidis</i> ), White grubs ( <i>Phyllophaga</i> spp., <i>Anomala</i> spp.), Cutworms ( <i>Agrotis</i> spp.), Thrips ( <i>Frankliniella</i> spp.), Lesser cornstalk borer ( <i>Elasmopalpus lignosellus</i> ).	

# APPENDIX IV

## Maize World Production

World Maize	Element			
	Seed (Mt)	Area Harvested (Ha)	Yield (Hg/Ha)	Production (Mt)
1961	6,223,099	105,484,151	19,435	205,004,683
1962	6,370,267	103,418,906	19,808	204,856,937
1963	6,193,721	108,384,382	20,319	220,228,333
1964	5,785,022	107,790,032	19,961	215,162,627
1965	5,988,088	106,591,240	21,252	226,524,256
1966	5,944,346	111,157,704	22,096	245,609,160
1967	5,872,917	112,313,038	24,266	272,538,473
1968	5,981,586	111,494,042	22,927	255,620,551
1969	5,838,480	111,242,302	24,226	269,491,068
1970	6,013,828	113,027,431	23,519	265,831,145
1971	6,185,867	118,150,571	26,544	313,622,622
1972	6,137,730	114,910,552	26,875	308,826,290
1973	6,132,362	116,856,034	27,238	318,290,469
1974	6,074,833	119,772,684	25,572	306,287,347
1975	6,429,594	121,442,141	28,133	341,656,971
1976	6,170,127	124,154,181	28,382	352,370,866
1977	6,181,283	125,192,168	29,679	371,561,355
1978	6,235,069	124,664,903	31,570	393,562,091
1979	6,281,256	123,598,634	33,866	418,577,993
1980	6,373,981	125,694,717	31,551	396,573,388
1981	6,440,288	127,816,716	34,950	446,722,107
1982	6,300,922	124,310,829	36,109	448,875,780
1983	6,605,234	117,763,540	29,468	347,024,034
1984	6,711,131	127,703,340	35,269	450,399,992
1985	6,646,135	130,454,042	37,214	485,474,301
1986	6,806,025	131,754,681	36,293	478,178,515
1987	6,623,584	129,888,090	34,880	453,054,894
1988	7,013,976	129,902,556	31,019	402,940,593
1989	7,158,041	131,711,470	36,203	476,833,660
1990	7,090,222	131,315,568	36,801	483,248,513
1991	7,379,181	134,125,220	36,851	494,267,664
1992	5,487,753	136,974,563	38,945	533,443,038
1993	5,497,737	131,500,199	36,242	476,576,466
1994	5,360,864	138,334,591	41,139	569,095,143
1995	5,474,640	136,271,016	37,914	516,655,836
1996	5,691,964	139,856,300	42,127	589,171,299
1997	5,588,723	141,270,173	41,407	584,954,064
1998	5,788,484	138,816,826	44,308	615,063,554
1999	5,765,380	138,460,288	43,786	606,261,782
2000	5,722,092	138,738,942	42,742	592,999,083
2001	5,912,420	137,596,759	44,273	609,181,620

Source : FAOSAT <http://apps.fao.org>

## SECTION 4 OILSEED RAPE (*BRASSICA NAPUS* L.)

### 1. General Information

This consensus document addresses the biology of the species *Brassica napus* L. Included are general descriptions of this species as a crop plant, its origin as a species, its reproductive biology, its centres of origin, and its general ecology. The ecology of this species is not described in relation to specific geographic regions. Special emphasis has been placed on detailing potential hybridisation between *B. napus* and its close relatives, although this discussion is limited to hybridisation events which do not require intervention through means such as embryo rescue (*i.e.* these events could possibly occur in nature, and could result in fertile offspring).

This document was prepared by a lead country, Canada. It is based on material developed in OECD Member countries – for example, for risk assessments or for presentation at conferences and scientific meetings. It is intended for use by regulatory authorities and others who have responsibility for assessments of transgenic plants proposed for commercialisation, and by those who are actively involved in these plants' design and development.

The table in the Appendix showing potential interactions of *B. napus* with other life forms during its life cycle was developed with respect to Canada. As such, it is intended to serve as an example. Member countries are encouraged to develop tables showing interacting organisms specific to their own geographic regions and environments.

### 2. General Description and Use as a Crop

*Brassica napus* L. is a member of the subtribe *Brassicinae* of the tribe *Brassicaceae* of the Cruciferous (*Brassicaceae*) family, sometimes referred to as the mustard family. The name "cruciferous" comes from the shape of its flowers, which have four diagonally opposite petals in the form of a cross. The dark bluish green foliage of *B. napus* is glaucous, smooth or has a few scattered hairs near the margins, and is partially clasping. The stems are well branched, although the degree of branching depends on variety and environmental conditions; branches originate in the axils of the highest leaves on the stem, and each terminates in an inflorescence. The inflorescence is an elongated raceme; the flowers are yellow, clustered at the top but not higher than the terminal buds, and open upwards from the base of the raceme (Musil, 1950).

There are two types of *B. napus*: 1) oil-yielding oleiferous rape, of which one subset with specific quality characteristics is often referred to as "canola" (vernacular name), and 2) the tuber-bearing swede or rutabaga. This document is written for oil-yielding oleiferous rape. The oleiferous type can also be subdivided into spring and winter forms. Sanskrit writings of 2000 to 1500 BC directly refer to oleiferous *B. napus* forms (sarson types) and mustard. Greek, Roman and Chinese writings of 500 to 200 BC refer to rapiferous forms of *B. rapa* (Downey and Röbbelen, 1989). In Europe, domestication is believed to have occurred in the early Middle Ages. Commercial plantings of rapeseed are recorded in the Netherlands as early as the 16th century. At that time rapeseed oil was used primarily as an oil for lamps. Later it came to be used as a lubricant in steam engines.

Although used widely as an edible oil in Asia, only through breeding for improved oil quality, and the development of improved processing techniques, has rapeseed oil become important in western countries. Since the Second World War, rapeseed production in Europe and Canada has increased dramatically as a result of improved oil and meal quality. Modern techniques of plant transformation and genotype identification using isozymes, restriction fragment length polymorphism (RFLP) markers, or random amplified polymorphic DNA (RAPDs) markers will complement classical breeding for the production of other improved lines (Buzza, 1995). China, India, Europe and Canada are now the top producers, although this crop can be successfully grown in the United States, South America and Australia, where annual production has increased sharply over the last few years.

Today, two species of *Brassica* have commercialised varieties with "double low" characteristics, i.e. low erucic acid content in the fatty acid profile and very low glucosinolate content in the meal, characteristics desirable for high-quality vegetable oil and high-quality animal feed. In North America these species (*B. napus* and *B. rapa*) are considered to be of "canola" quality. *B. napus* is grown as a winter annual in regions where winter conditions do not result in very low temperatures, which would kill the plants. These biotypes typically require vernalisation before the onset of stem elongation, raceme development, flowering and seed set. In North America and northern parts of Europe, a spring biotype of *B. napus* that requires no vernalisation prior to flowering is grown. These biotypes are typically lower yielding than the winter annual types, but require considerably less time to complete their life cycle.

### 3. Agronomic Practices for Oleiferous *B. napus*

The spring-type oleiferous *B. napus*, a cool season crop, is not very drought tolerant. It is widely adapted and performs well under a range of soil conditions, provided that moisture and fertility levels are adequate. Air and soil temperatures influence plant growth and productivity. The optimum temperature for maximal growth and development of spring-type oilseed rape is just over 20°C, and it is best grown between 12°C and 30°C. After emergence, seedlings prefer relatively cool temperatures up to flowering; high temperatures at flowering will hasten the plant's development, reducing the time from flowering to maturity. Among cultivated crop plants, *Brassica* species show the highest nutritional demand for sulphur.

Due to increased awareness of soil conservation issues, minimal or no-till *B. napus* production is advised, in which most of the crop residue and stubble are left on the soil surface to trap snow, reduce snow melt run-off, reduce wind and water erosion of the soil, and increase soil water storage. Reduced tillage techniques, however, are only effective when combined with a good systematic weed control programme. Winter oilseed rape covers the soil for ten to eleven months. It has high nutritional demands in autumn and reduces soil erosion in winter.

Weeds can be one of the most limiting parameters in rapeseed production. The closely related cruciferous weeds, for example wild mustard (*Sinapis arvensis*), stinkweed (*Thlaspi arvense*), shepherd's purse (*Capsella bursa-pastoris*), ball mustard (*Neslia paniculata*), flixweed (*Descurainia sophia*), wormseed mustard (*Erysimum cheiranthoides*), hare's ear mustard (*Coringia orientalis*), common peppergrass (*Lepidium densifolium*), etc., are often problematic. Spring-type oilseed rape does not compete well with weeds in the early growth stages, as it is slow-growing and slow to cover the ground. Weeds must be controlled early to avoid yield loss due to competition. Although rapeseed crops can be attacked by a number of insect pests, insect control must be carefully designed to reduce unnecessary and costly pesticide applications, the chances of resistance build-up in insects, and damage to honeybees and native pollinating insects. Diseases can be severe in large production areas, and are greatly influenced by cultivation practices and environmental factors, so that disease management programmes are advisable (refer to the table in the Appendix for examples of *B. napus* pests and diseases in Canada).

When the first siliques begin to shatter, *B. napus* can be cut just below the level of the seed pods and swathed. The use of dessicants allows a reduction of shattering, and possibly allows direct combining.

Generally, oilseed rape should not be grown on the same field more often than once every three to four years in order to prevent the build-up of diseases, insects and weeds. Chemical residues from herbicides and volunteer growth from previous crops (including rapeseed crops grown for different oil types) are also important factors to consider when selecting sites, although suitable soil treatments following harvest may considerably reduce the volunteer problem.

#### 4. Centres of Origin/Diversity<sup>8</sup>

##### A. Geographic origin of *B. napus*

The origins of *B. napus* (an amphidiploid with chromosome  $n=19$ ) are obscure, but were initially proposed to involve natural interspecific hybridisation between the two diploid species *B. oleracea* ( $n = 9$ ) and *B. rapa* (syn. *campestris*)<sup>9</sup> ( $n = 10$ ) (U, 1935). Recent evidence (Song and Osborn, 1992), through analyses of chloroplast and mitochondrial DNA, suggests that *B. montana* ( $n = 9$ ) might be closely related to the prototype that gave rise to both cytoplasm of *B. rapa* and *B. oleracea*. It also suggests that *B. napus* has multiple origins, and that most cultivated forms of *B. napus* were derived from a cross in which a closely related ancestral species of *B. rapa* and *B. oleracea* was the maternal donor. In Europe, it is predominantly the winter form which has become a common yellow crucifer found along roadsides, on waste sites and cultivated ground, on docks, in cities and towns, on tips, and on arable fields and along riverbanks. In the British Isles, it has been naturalised wherever oilseed rape is grown. It is a relatively recent introduction into Canada and the United States, and is described as an occasional weed, escape or volunteer in cultivated fields (Munz, 1968, and Muenscher, 1980). It is found typically in crops, fields and gardens, along roadsides, and on waste sites.

##### B. Geographic origin of *B. oleracea*

The wild form of *B. oleracea*, a suffrutescent (low, shrubby plant with woody lower parts of stems and herbaceous upper parts) perennial, grows along the coast of the Mediterranean from Greece through to the Atlantic coasts of Spain and France, around the coast of England, and to a limited extent in Helgoland (Snogerup *et al.*, 1990). Typically the wild type is found on limestone and chalk cliffs in situations protected from grazing. Individuals are often found below cliffs in scree, where they grow among other shrubs, and some populations are found on steep grassy slopes. In Helgoland, populations are found on open rocky ground. In Europe and North America, domesticated types have been reported as escapes but do not form self-sustaining populations outside cultivation. *B. oleracea* is a recent introduction into North America.

##### C. Geographic origin of *B. rapa*

Wild *B. rapa* (subspecies *sylvestris* L.) is regarded as the species from which the ssp. *rapa* (cultivated turnip) and *oleifera* (turnip rape) originated. It is native throughout Europe, Russia, central Asia and the Near East (Prakash and Hinata, 1980), with Europe proposed as one centre of origin. There is some debate as to whether the Asian and Near Eastern types arose from an independent centre of origin in Afghanistan

8. This section draws heavily on discussions with, and a review paper prepared by, Dr S.I. Warwick and A. Francis (1994), Centre for Land and Biological Resources Research, Agriculture and Agri-Food Canada.

9. First described as two species by Linnaeus, with *B. rapa* being the turnip form and *B. campestris* the oleiferous form. Metzger in 1933 concluded that these were the same species and chose the name *B. rapa* (Toxepous *et al.*, 1984).

and then moved eastward as *B. rapa* became domesticated. Prakash and Hinata (1980) suggest that oleiferous *B. rapa* subspecies developed in two places, giving rise to two different races, one European and the other Asian.

Typically, *B. rapa* is found in coastal lowlands, high montane areas (the slopes of high valleys or mountain ranges), and alpine and high sierras. In Canada, where it is a recent introduction, it is found on disturbed land, typically in crops, fields and gardens, along roadsides, and on waste sites (Warwick and Francis, 1994).

#### **D. Geographic origin of *B. montana***

*B. montana*, possibly a progenitor species of *B. napus* (see above) and also a suffrutescent perennial, originates in the Mediterranean coastal area between Spain and Northern Italy (Snogerup *et al.*, 1990). It is found typically on or below limestone cliffs and rocks, walls, etc., often on disturbed ground. Although usually found in coastal areas and on rocky islets, it has been recorded at an elevation of 1000 m somewhat inland of the coast.

#### **5. Reproductive Biology**

Under field conditions the fertilisation of ovules usually results from self-pollination, although outcrossing rates of 5-30 per cent have been reported (Hühn and Rakow, 1979, and Rakow and Woods, 1987). The pollen, which is heavy and sticky, can be transferred from plant to plant through physical contact between neighbouring plants and by wind and insects. Oilseed rape pollen has been detected in the air above rape fields (Williams, 1984) and beyond the borders of a rape crop (Olsson, 1955); however, the concentration decreases rapidly with increasing distance from the source of the pollen and windborne pollen may make no or only a negligible contribution to long-distance pollination of oilseed rape (Mesquida and Renard, 1982, and McCartney and Lacey, 1991). Timmons *et al.*, (1995), using pollen traps and “bait” plants whose petals had been removed and which had been emasculated, reported airborne pollen at distances up to 2.5 km from commercial plantings of *B. napus*. The “bait” plants also produced some seed at this distance from the commercial oilseed rape, suggesting the airborne pollen might be capable of successful fertilisation events.

Pollinating insects, in particular honeybees (*Apis mellifera*) and bumblebees (*Bombus* sp.), play a major role in *B. napus* pollination and are believed to be involved in the transfer of pollen over long distances. Oilseed rape is very attractive to bees because it produces large quantities of nectar and pollen. Williams *et al.*, (1987) reported that “plants in plots caged with bees had their flowers pollinated faster, shed petals sooner, finished flowering earlier and were shorter than plants caged without bees.” *B. napus* pollen is a major food source for bees, and hives are often placed near rapeseed fields during flowering to take advantage of the honey production potential (Marquard and Walker, 1995).

When beehives were placed at the centre of each side of a 1 ha square of non-transgenic *B. napus* plants with a 9 m circle of transgenic plants at the centre, Scheffler *et al.*, (1993) reported outcrossing ranging from 1.5 per cent at 1 m to 0.00033 per cent at 47 m. In a later study using 20 x 20 m plots of transgenic and non-transgenic plants, separated by distances of 200 and 400 m, the space separating the plots being either bare ground or planted with barley (*Hordeum vulgare*), Scheffler *et al.*, (1995) reported the average frequency of hybridisation to be 0.0156 per cent at 200 m and 0.0038 per cent at 400 m.

The dynamics of bee-mediated pollen movement depend on the quantity of pollen available (size and density of donor population) and the size and location of the receiving populations, as well as on environmental conditions and insect activity (Levin and Kerster, 1969, Ellstrand *et al.*, 1989, and Klinger *et al.*, 1992). These studies, together with the findings of Scheffler *et al.*, (1993 and 1995), suggest that

surrounding an experimental plot of *B. napus* with other plants of the same species flowering synchronously with the experimental plants could decrease the long-distance dispersal of pollen from experimental plants by insects.

## 6. Cultivated *B. napus* as a Volunteer Weed

As with all crops cultivated and harvested at the field scale, some seed may escape harvesting and remain in the soil until the following season, when it germinates either before or following the seeding of the succeeding crop. In some instances the volunteers may give considerable competition to the seeded crop and cause deterioration in the quality of the crop harvest. In such instances, chemical and/or mechanical control is essential.

The problem of volunteer plants in succeeding crops is common to most field crop species. Much depends on the management practices used in the production of the crop, for example whether the plants have disbursed seed at the time of harvest, the setting of the harvesting equipment, and the speed of the harvesting operation, which will determine whether more or less seed is lost by the harvester. With crops of the *Brassica* family, because of the small seed size and large number of seeds produced by the crop, poor management practices can result in severe volunteer problems in succeeding crops. Suitable soil treatment after the harvest can considerably reduce the volunteer problem.

## 7. Crosses

### A. Inter-species/-genus

In considering potential environmental impact following the unconfined release of genetically modified *B. napus*, it is important to have an understanding of the potential for the development of hybrids through interspecific and intergeneric crosses between the crop and its related species. The development of such hybrids could result in the introgression of the novel traits into these related species, and result in:

- The related species becoming weedy or more invasive of natural ecosystems.
- Altered environmental interactions, potentially causing harm to the environment or to human health and safety.

While many interspecific and intergeneric crosses have been made between *B. napus* and its relatives (Prakash and Hinata, 1980, Warwick and Black, 1993, and Scheffler and Dale, 1994), many have necessitated intervention in the form of ovary culture, ovule culture, embryo rescue and protoplast fusion. Reported in Table 1.3, and ranked in order of relative ability to form hybrid progeny when crossed with *B. napus*, are instances reported by Scheffler and Dale (1994) of sexually obtained interspecific and intergeneric crosses with *B. napus*.

Flowering periods of *B. napus* and these species are critical. For interhybridisation events to occur, their flowering periods, which are largely environmentally influenced, must overlap at least partially. To evaluate hybridisation potential, it is important to know the flowering chronology of both the cultivated plant and related species; the physical distance between potentially hybridising species; occurrence of vectors for pollination; and how pollination takes place.

The chromosome numbers of the cultivated species and relatives are also important. Many hybrids fail to occur due to lack of development of the endosperm (tissue resulting from the fertilisation of the two polar nuclei of the embryo sac by a male reproductive nucleus). The ratio of maternal and paternal chromosomes must be of 2:1 or higher (Nishiyama and Inomata, 1966). This explains why the direction of

crossing is often important. The pollination of a tetraploid female parent by a diploid male usually produces seeds. The reciprocal cross, on the other hand, is sterile. In order to understand existing exceptions, Johnston *et al.*, (1980) proposed the concept of the endosperm balance number (EBN), where the value attributed to a given species is not linked to its chromosome number but to an arbitrary value determined from a successful cross and from the hypothesis that the EBN ratio is 2:1 in the endosperm.

**Table 1.3 Sexually obtained interspecific and intergeneric crosses with *B. Napus* (reported by Scheffler and Dale, 1994)**

Cross female x male	Progeny	References
<i>B. rapa</i> x <i>B. napus</i>	SH, F1, F2, BcP	Morinaga, 1929 U and Nagamatsu, 1933 U, 1935 Bing <i>et al.</i> , 1991 Jørgensen and Andersen, 1994
<i>B. napus</i> x <i>B. rapa</i>	SH, F1, F2, BcP	Mikkelsen <i>et al.</i> , 1996 Morinaga, 1929 U and Nagamatsu, 1933 U, 1935 Bing <i>et al.</i> , 1991 Jørgensen and Andersen, 1994
<i>B. juncea</i> x <i>B. napus</i>	SH, F1, F2, BcP	Mikkelsen <i>et al.</i> , 1996 Morinaga, 1934 Roy, 1980 Bing <i>et al.</i> , 1991 Fernandez-Serrano <i>et al.</i> , 1991
<i>B. napus</i> x <i>B. juncea</i>	SH, F1, F2, BcP	Frello <i>et al.</i> , 1995 Morinaga, 1934 Roy, 1980 Bing <i>et al.</i> , 1991 Fernandez-Serrano <i>et al.</i> , 1991
<i>B. oleracea</i> x <i>B. napus</i>	F1	Frello <i>et al.</i> , 1995 U, 1935
<i>B. napus</i> x <i>B. oleracea</i>	F1, F2, BcP	Roemer, 1935 Röbbelen, 1966 Yamagishi and Takayanagi, 1982
<i>B. carinata</i> x <i>B. napus</i>	F1, F2, BcP	Roy, 1980 Fernandez-Escobar <i>et al.</i> , 1988 Fernandez-Serrano <i>et al.</i> , 1991
<i>B. napus</i> x <i>B. carinata</i>	F1, F2, BcP	U, 1935 Roy, 1980 Fernandez-Escobar <i>et al.</i> , 1988 Fernandez-Serrano <i>et al.</i> , 1991
<i>B. nigra</i> x <i>B. napus</i>	SH, F1, BcP	Bing <i>et al.</i> , 1991
<i>B. napus</i> x <i>B. nigra</i>	SH, F1, F2, BcP	Heyn, 1977 Bing <i>et al.</i> , 1991
<i>B. napus</i> x <i>Hirschfeldia incana</i>	SH, SH(BnMS), F1, BcP	Lefol <i>et al.</i> , 1991 Chevre <i>et al.</i> , 1992 Eber <i>et al.</i> , 1994
<i>B. napus</i> x <i>Raphanus raphanistrum</i>	SH, SH(BnMS), F1, BcP	Chevre <i>et al.</i> , 1992 Lefol <i>et al.</i> , <i>in press</i> Eber <i>et al.</i> , 1994
<i>Diplotaxis erucoides</i> x <i>B. napus</i>	F1, BcP	Ringdahl <i>et al.</i> , 1987
<i>D. muralis</i> x <i>B. napus</i>	F1, BcP	Ringdahl <i>et al.</i> , 1987
<i>B. napus</i> x <i>Erucastrum gallicum</i> *	F1, BcP	Lefol <i>et al.</i> , <i>in press</i>
<i>B. napus</i> x <i>Sinapis alba</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>S. arvensis</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>B. fruticulosa</i>	F1	Heyn, 1977

<i>B. napus</i> x <i>B. tournefortii</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>D. tenuifolia</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>Eruca sativa</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>R. rugosum</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>R. sativus</i>	F1	McNaughton and Ross, 1978

Note:

SH = spontaneous hybrids formed without the aid of emasculation and manual pollination transfer;

SH(BnMS) = spontaneous hybrids with male sterile *B. napus* as female parent;

F1 = F1 hybrids produced through intervention of some sort, i.e. emasculation and manual pollination;

F2 = F2 hybrids produced;

BcP = backcross progeny produced.

\* This hybridisation event not reported by Scheffler and Dale (1994)

Generally, crosses between two species are possible only if the female species has a polyploidy level at least as high as the pollinating male species. Since *B. napus* is tetraploid, it will cross more readily with wild species (diploid) as a female parent (Sikka, 1940, Harberd and McArthur, 1980, and Kerlan *et al.*, 1991). In the case of *Raphanus raphanistrum*, no difference was noted in the direction of crosses (Kerlan *et al.*, 1991); in the case of *Sinapis alba*, the opposite situation occurs (Ripley and Arnison, 1990).

For a trait to become incorporated into a species genome, recurrent backcrossing of plants of that species by the hybrid intermediaries, and survival and fertility of the resulting offspring, will be necessary.

## B. Introgression into relatives

The potential hybridisation events listed are intended to assist the assessment of the potential for introgression of "novel traits" introduced from cultivated *B. napus* into wild relatives. The first step in this assessment is to determine which, if any, of the potential "mates" of *B. napus* are recorded as present in the geographic region where the cultivation is proposed. Should there be potential wild relative "mates" present, the frequency of hybridisation events and the potential for environmental impact should introgression occur would then be considered. Should a trait with positive selective value be introgressed into wild or weedy populations, the gene may become a permanent part of the gene pool of these populations.

The above listed species are all plants of "disturbed land" habitats. Their success will be dependent on their ability to compete for space with other primary colonisers, particularly other successful weedy plant types. This in turn will depend on how well suited they are to the particular climate, soil conditions, etc. of individual sites. Equal ability of the hybrids to compete among wild populations or in cultivated fields has been shown for *B. napus* and hybrids (Lefol *et al.*, 1995).

## C. Interactions with other organisms

The table in the Appendix is intended as an identification guide for categories of organisms which interact with *B. napus*. This table, representative of Canada, is intended to serve as an example only. Environmental safety assessors should, on a country-by-country basis, draw up their own lists as a guide for assessing potential effects of the release of genetically modified plants on interacting organisms in their country.

## 8. Ecology

*B. napus* and its progenitors grow in "disturbed land" habitats. In non-managed ecosystems these species may be considered "primary colonisers," i.e. plant species that are the first to take advantage of the

disturbed land, where they compete for space with plants of similar types. Unless the habitats are disturbed on a regular basis, for example along the edges of cliffs, rivers, and pathways, populations of these types of plants will be displaced by intermediaries and finally by plants that form climax ecologies, such as perennial grasses on prairies and tree species and perennial shrubs in forests.

In non-natural ecosystems, including along roadsides and on industrial and waste sites as well as cropland, there is potential, because of their "primary colonising" nature, for ever-present populations of these species to be maintained. It is in such habitats that the species are recorded among the flora of countries where *B. napus* has been introduced as a crop plant. Their success will depend on their ability to compete for space with other primary colonisers, in particular successful weedy types. This, in turn, will depend on how well suited they are to the particular climate, soil conditions, etc. of individual sites.

In crop production systems, poor management practices and insufficient resistance to pod shattering may result in large amounts of *B. napus* seed not being harvested. Especially where there are high crop densities, this may cause volunteer "weed" problems in succeeding crops as well as contamination of such crops with respect to their seed quality.

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## APPENDIX

Potential Interactions of *B. napus* with other life forms during its life cycle (Canada)X indicates the type of interaction between the listed organisms and *B. napus*

Other life forms	Interaction with <i>B. napus</i>			
	Pathogen	Symbiont or beneficial organism	Consumer	Gene transfer
<i>Albugo candida</i>	X			
<i>Alternaria</i> spp.	X			
<i>Botrytis cinerea</i>	X			
<i>Erysiphe</i> spp.	X			
<i>Leptosphaeria maculans</i>	X			
<i>Peronospora parasitica</i>	X			
<i>Plasmiodiophora brassicae</i>	X			
<i>Pseudocercospora capsellae</i>	X			
<i>Pseudomonas</i> sp.	X			
<i>Pyrenopeziza brassicae</i>	X			
<i>Pythium debaryanum</i>	X			
<i>Rhizoctonia solani</i>	X			
<i>Sclerotinia sclerotiorum</i>	X			
<i>Xanthomonas</i> spp.	X			
<i>Verticillium dahliae</i>	X			
Mychorrhizal fungi		X		
Aster yellow mycoplasma	X			
Cauliflower Mosaic Virus (CaMV)	X			
Beet Western Yellow Virus (BWYV)	X			
Turnip mosaic virus	X			
Soil microbes		X		
Earthworms		X		
Flea beetle			X	
Pollinators		X	X	
Soil insects			X	
Animal browsers (e.g. deer, hare, rabbit)			X	
Birds			X	
Other <i>Brassica napus</i>				X
<i>Brassica rapa</i>				X
<i>Brassica juncea</i>				X
<i>Brassica nigra</i>				X
<i>Raphanus raphanistrum</i>				X
<i>Erucastrum gallicum</i>				X
Others				X

## SECTION 5

### RICE (*ORYZA SATIVA* L.)

#### 1. Use as a Crop Plant

Rice is grown worldwide and is a staple food for about a half of the world's population. It is a nutritious grain crop which contains carbohydrates, proteins, lipids, minerals, etc. Rice straw is an important animal feed in many countries.

Rice is now cultivated as far north as the banks of the Amur River (53° N) on the border between Russia and China, and as far south as central Argentina (40° S) (IRRI, 1985). It is grown in cool climates in the mountains of Nepal and India, and under irrigation in the hot deserts of Pakistan, Iran and Egypt. It is an upland crop in parts of Asia, Africa and Latin America. At the other environmental extreme are floating rices, which thrive in seasonally deeply flooded areas such as river deltas - the Mekong in Vietnam, the Chao Phraya in Thailand, the Irrawady in Myanmar, and the Ganges-Brahmaputra in Bangladesh and eastern India, for example. Rice can also be grown in areas with saline, alkali or acid-sulphate soils. Clearly, it is well adapted to diverse growing conditions.

There are two cultivated rice species: *Oryza sativa*, grown worldwide, and *Oryza glaberrima*, grown in West and Central Africa. *O. sativa* has many ecotypes (cultivars) adapted to various environmental conditions. The morphology, physiology, agronomy, genetics and biochemistry of *O. sativa* have been intensively studied over a long period.

#### 2. Taxonomic Status

The genus *Oryza* contains 22 species: two are cultivated and 20 are wild (Table 2.4) (Morishima, 1984; Vaughan, 1994). *O. sativa* is cultivated worldwide, and the word "rice" generally indicates a plant and a crop of this species. *O. glaberrima* is cultivated in West and Central Africa.

The basic chromosome number of the genus *Oryza* is 12. *O. sativa*, *O. glaberrima* and 14 wild species are diploids with 24 chromosomes, and eight wild species are tetraploids with 48 chromosomes. *O. punctata* consists of diploid and tetraploid types. Genome symbols, A to F, are assigned to the species on the basis of meiotic chromosome pairing of F<sub>1</sub> hybrids. Those species with the same genome symbols show no significant disturbance in chromosome pairing in their hybrids. Recently Aggarwal *et al.*, (1997) used molecular methods to identify genomes G, H and J.

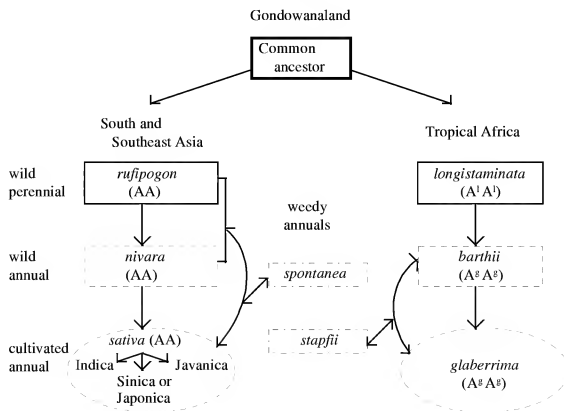
The progenitors of *O. sativa* are considered to be the Asian AA genome diploid species *O. nivara* and *O. rufipogon*, and those of *O. glaberrima* to be the African AA genome diploid species *O. barthii* and *O. longistaminata* (Figure 1.2) (Chang, 1976).

Table 1.4 Species belonging to the genus *Oryza*

Species	Number of chromosomes	Genome	Geographical distribution
Section <i>Oryzae</i>			
[ <i>O. sativa</i> complex]			
<i>O. sativa</i> L.	24	AA	Worldwide, cultivated
<i>O. nivara</i> Sharma et Shastry	24	AA	Asia
<i>O. rufipogon</i> Griff.	24	AA	Asia, Australia, America (Latin, South)
<i>O. glaberrima</i> Steud.	24	AA	Africa, cultivated
<i>O. barthii</i> A. Chev.	24	AA	Africa
<i>O. longistaminata</i> Chev. et Roehr.	24	AA	Africa
<i>O. meridionalis</i> Ng.	24	AA	Australia
[ <i>O. officinalis</i> complex]			
<i>O. officinalis</i> Wall. ex Watt	24	CC	Asia, New Guinea
<i>O. minuta</i> Presl. et Presl.	48	BBCC	Asia, New Guinea
<i>O. eichingeri</i> Peter	24	CC	Africa, Asia (Sri Lanka)
<i>O. rhizomatis</i> Vaughan	24	CC	Asia (Sri Lanka)
<i>O. punctata</i> Kotschy ex Steud.	24,48	BB,BBCC	Africa
<i>O. latifolia</i> Desv.	48	CCDD	America (Latin, South)
<i>O. alta</i> Swallen	48	CCDD	America (South)
<i>O. grandiglumis</i> Prod.	48	CCDD	America (South)
<i>O. australiensis</i> Domin	24	EE	Australia
Section <i>Ridleyanae</i>			
<i>O. brachyantha</i> Chev. et Roehr.	24	FF	Africa
<i>O. schlechteri</i> Pilger	48		New Guinea
[ <i>O. ridleyi</i> complex]			
<i>O. ridleyi</i> Hook. f.	48	HHJJ	Asia, New Guinea
<i>O. longiglumis</i> Jansen	48	HHJJ	New Guinea
Section <i>Granulatae</i>			
[ <i>O. meyeriana</i> complex]			
<i>O. meyeriana</i> Baill.	24	GG	Asia
<i>O. granulata</i> Nees et Arn. ex Watt	24	GG	Asia

Source : Morishima, 1998; Vaughan, 1994; Aggarwal *et al.*, 199

Figure 1.2 Evolutionary pathway of the two cultivated species of rice



Source : Adapted from Chang, 1976

### 3. Centre of Origin/Diversity

The genetic diversity of various traits in local cultivars of rice is greatest in the area extending from Assam in India and Bangladesh to Myanmar and northern Thailand, and to Yunnan Province in China (Oka, 1988). This area is characterised by topographical and hydrological heterogeneity, and is considered the centre of diversity. Today genetic diversity in this area is being lost, since many rice growers are now growing modern cultivars.

The wild progenitors of *Oryza sativa* are the Asian common wild rices, which show a wide range of variation from perennial to annual types.

Domestication of Asian rice, *O. sativa*, is considered to have occurred in 15,000 to 10,000 BC. Annual forms might have gradually developed in northeastern and eastern India, northern Southeast Asia and southern China (Chang, 1985). They spread and diversified to form two ecological groups, Indica and Japonica (Oka, 1988). There are other studies indicating that the two groups were derived independently from the domestication of two divergent wild rices in Southeast Asia and China, respectively (Second, 1982; 1986).

The wild progenitors of African cultivated rice, *O. glaberrima*, are grasses endemic to West Africa. *O. glaberrima* is considered to have been domesticated in the Niger River delta (Chang, 1976). The primary centre of diversity of *O. glaberrima* is the swampy basin of the upper Niger. In rice fields managed by West African farmers, *O. sativa* and *O. glaberrima* are sometimes grown as mixtures of varying proportions (Chang, 1976; Oka *et al.*, 1978; Morishima and Oka, 1979).

#### 4. Identification Methods

##### A. General description of *Oryza sativa*

Coleoptiles and roots first emerge from the germinating rice seeds. Seedlings differentiate leaves from the growing point of the main culm and tiller buds in the axil of leaves. Panicle primordia differentiate at the top of culms. At heading time, panicles come out of flag-leaf sheaths. Flowering takes place in spikelets on a panicle, followed by pollination on stigmata and fertilisation in ovules. Embryo and endosperm mature in the ovule and become a seed for the next generation. Rice plants are very easily propagated by seeds or tiller buds.

The leaf consists of a blade, a sheath, and a ligule and auricle at the junction between blade and sheath. The culm consists of nodes and hollow internodes. The spikelet has six stamens and the ovary has a two-branched stigma. The seed consists of embryo, endosperm, pericarp and testa enclosed by a palea, and a lemma with an apiculus on the top of the lemma.

##### B. Identification among cultivars of *O. sativa*

There are a great number of rice cultivars grown in the world. More than 100,000 accessions are conserved in national and international genebanks such as that of the International Rice Research Institute.

Cultivars can be distinguished by differences in morphological, physiological and ecological characters. Essential characters for identifying cultivars are adaptation to different water regimes; growing habit; plant height; shape, size and colour of culm, leaf blade, panicle, hull, apiculus and dehulled grain; presence or absence of pubescence; grain aroma; growth duration, including time to heading and maturity; resistance or tolerance to disease and insect pests, temperature, lodging, grain shattering, seed germinability and seed dormancy; grain quality, including appearance, starch glutinousness and protein content. For rice growers, the cultivar's adaptation to water regimes is the most important consideration, followed by grain characters such as glutinous or non-glutinous, then whether the cultivar is early or late maturing, and other characteristics.

##### C. Identification among groups of *O. sativa*

*O. sativa* has been classified into several groups on the basis of morphological, physiological and ecological characters. Kato *et al.*, (1928) reported two subspecies, *japonica* and *indica*, from the sterility of  $F_1$  hybrids between cultivars. Ting (1949, 1957) proposed that the subspecies *indica* and *japonica* corresponded to the *hsien* and *keng* classification in China. Matsuo (1952) classified world rice cultivars into group A, having round grains like those of Japanese cultivars; B, having large grains like some tropical cultivars; and C, having slender grains like Indica cultivars. Oka (1958) classified diverse varietal types into Indica and Japonica. Indica cultivars are distributed mainly in the tropical to subtropical zones, while Japonica cultivars are grown in the tropical to northern temperate zones. The two groups differ in many characters when typical varieties are compared, but they show some overlapping variations in each character. Oka (1988) further classified the Indica group into seven sub-groups (Boro, Aus, Broadcast Aman, Transplanted Aman, Rayada, Ashina and Hill Rice) and the Japonica group into tropical and temperate subgroups. The name Javanica was originally used for tropical Japonica-like varieties from Java, and the morphological and physiological traits of currently cultivated Asian and American Javanica fall exactly in the Japonica group (Glaszmann and Arrauadeau, 1986; Sato, 1987; Oka, 1988).

Traditionally, the shape or length/width ratio of the spikelet (unhulled rice), and cereal chemistry characteristics such as the hardness and stickiness of cooked rice, have been regarded as criteria to distinguish between Indica and Japonica cultivars. Indica cultivars have longer grains, and are harder and much less sticky when cooked than Japonica. However, this determining characteristic is occasionally

unreliable because of overlapping variation between the two groups. Indica and Japonica are the group names for cultivars that have been selectively adapted for physiological differences favouring different ecological niches.

A discriminant formula combining the measurements of potassium chlorate resistance, low-temperature sensitivity, drought resistance, apiculus hair length and phenol reaction of unhulled rice can classify those two groups efficiently (Morishima and Oka, 1981). Potassium chlorate resistance has the highest diagnostic power to identify each group, followed by drought resistance, apiculus hair length and cold sensitivity score.

Isozyme patterns are effective for identifying cultivar groups. Glaszmann (1987) grouped local cultivars from different Asian countries into six groups, using 15 isozyme loci for eight enzymes detected in young seedlings. When other classifications were compared with these results, most of the cultivars were classified as Indica rice belonged to groups I and II, while group VI corresponded to the Japonica including both the temperate and tropical types. Further, groups III, IV and V included such cultivars as the Rayada rices of Bangladesh, the Sadri rices of Iran, and the Basmati rices of Pakistan and India, but these groups are not identifiable as Indica or Japonica. Kochko (1987a, b) reported isozyme patterns representative of Indica and Japonica groups in traditional cultivars from most African countries.

#### D. Differentiation between *O. sativa* and *O. glaberrima*

There are discrete differences between the key characters of *O. sativa* and *O. glaberrima* (Table 1.5), and intermediate type plants rarely exist (Morishima *et al.*, 1962). *O. sativa* has more secondary branches on the panicles, and longer and smoother ligules, than *O. glaberrima*. A typical *O. glaberrima* has glabrous (hairless) spikelets and leaf blades, while *O. sativa* cultivars are mostly pubescent, although most cultivars in the United States are glabrous. The seed of *O. glaberrima* has longer dormancy than that of *O. sativa*. *O. sativa* is cultivated as an annual agricultural crop, but botanically it is a perennial plant, while *O. glaberrima* is annual both botanically and agronomically. Alone, any of these traits cannot always be a definite discriminant of the two species.

**Table 1.5 Comparison of main characters of domesticated cultivars of *O. sativa* and *O. glaberrima***

Character	<i>O. sativa</i>	<i>O. glaberrima</i>
Habit	Essentially perennial	Annual
Ligule	Long and soft	Short and tough
Panicle branches	Many	Few
Frequency of glabrous varieties	Low	High
Varietal differentiation	Highly variable	Limited variation
Ecotypes	Many	Few
Distribution	Worldwide	Endemic to West Africa

Source : Modified from Oka, 1991

#### E. Identification of wild species

The wild progenitors of *O. sativa* are the Asian common wild rices, which show a wide variation from perennial to annual types. Wild species are taxonomically identified by examination of their key characters.

In the field, species are usually identified visually based on a combination of characteristics. On the basis of morphological and ecological data, multivariate analysis has been applied to classify wild plants into appropriate wild species groups (Morishima and Oka, 1960; Morishima, 1969).

Wild species are distinguished from *O. sativa* by such traits as habitat, plant type, colouration of spikelet and anther, length and shape of ligule and auricle, panicle type, and awnedness.

Isozyme patterns are also useful to distinguish wild species from *O. sativa*. *O. rufipogon*,<sup>10</sup> the wild species very closely related to *O. sativa*, possesses more alleles at different isozyme loci and is more polymorphic than *O. sativa* cultivars (Oka, 1988). However, the isozyme alleles from the Japonica type are found with a high frequency only in Chinese strains of *O. rufipogon*, while the alleles characterising the Indica type are observed predominantly in South Asian strains (Second, 1986). Oceanian *O. rufipogon* and *O. longistaminata* present a large genetic diversity of isozymes distinguishable from *O. sativa* and *O. glaberrima* (Second, 1986).

A general description of the morphology of wild species is included in Appendix I.

#### F. Genetic and molecular identification

It is possible to distinguish between cultivars of *O. sativa* and between *Oryza* species using genetic, cytological and molecular techniques.

##### *Gene linkage groups*

The data on the linkage maps of all identified genes concerning morphological and physiological traits on the 12 rice chromosomes are reported annually in the *Rice Genetic Newsletter* by the Committee of Gene Symbolisation, Nomenclature and Linkage Groups of the Rice Genetics Cooperative (Rice Genetics Cooperative, 1995). Prior to the work of the Rice Genetics Cooperative, it was difficult to compare results from different laboratories. The Rice Genetics Cooperative has developed an international standard for rice genetic studies.

There has been little research on *O. glaberrima* linkage maps, but the important characters are at the same locations as in *O. sativa* (Sano, 1988).

##### *DNA marker linkage maps*

More recently, DNA markers such as RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNAs) have been used to detect DNA polymorphism, which enables cultivars to be identified. Progress in mapping genes using DNA markers such as RFLP has also been reported (McCouch and Tanksley, 1991), and is updated and listed in the *Rice Genetics Newsletter*. Cultivars will be identified in the future on the basis of specific genes at defined locations on the rice genomes.

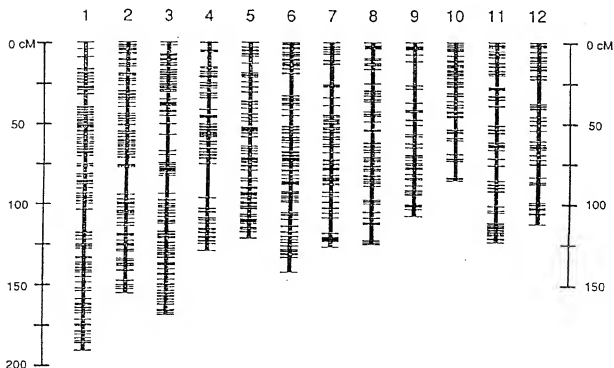
The DNA markers densely mapped on the linkage maps are powerful tools for precise analysis of genotypes of rice plants. Construction of genetic linkage maps using DNA markers such as RFLP and RAPD is in progress not only for *O. sativa*, but also for some wild species. A DNA linkage map has been developed consisting of about 1,400 DNA markers, along with about 1,500 cM over 12 rice chromosomes from an intraspecific cross of *O. sativa* (Figure 1.3) (Kurata *et al.*, 1994). From an interspecific backcross

10. The species name *O. rufipogon* used by Oka and his co-researchers may have included the annual species *O. nivara* and the perennial species *O. rufipogon* in Table 1.

between *O. sativa* and *O. longistaminata*, a molecular map has been constructed consisting of 726 markers for 12 chromosomes (Causse *et al.*, 1994). Some of the genes controlling morphologically, physiologically and agronomically important traits have been located on the linkage map.

Mapping of qualitative and quantitative trait loci has progressed rapidly in rice as a consequence, and DNA fingerprinting using RFLP and microsatellite markers will enable identification of individual plants, cultivars and species in the future.

**Figure 1.3 Rice RFLP linkage map constructed with 1,383 DNA markers**



Source : Modified by the National Institute of Agrobiological Resources (Japan) from Kurata *et al* (1994).

## 5. Reproductive Biology

### A. Sexual reproduction

*Oryza sativa* is basically an autogamous plant propagating through seeds produced by self-pollination. Fertilisation occurs in a spikelet, which has six anthers with more than 1,000 pollen grains in each, and an ovule with a branched stigma. Immediately after the spikelet opens at flowering, pollen is dispersed and germinates on the surface of the stigma. Only one pollen tube reaches an ovule to initiate double fertilisation.

The maturation of pollen in an anther is synchronised with the maturation of the ovule within the same spikelet. Pollen can maintain germinability only for several minutes after being shed from the anther under favourable temperature and moisture conditions, while ovules keep their viability to receive pollens for several days after maturation. Pollen of cultivated rice loses its viability within three to five minutes, but wild rice pollen has a longevity of up to nine minutes (Koga *et al.*, 1971; Oka and Morishima, 1967).

Most of the wild species have a larger and longer stigma which extends outside the spikelet, increasing the opportunity for outcrossing (Parmer *et al.*, 1979; Virmani and Edwards, 1983).

The degree of outcrossing is generally higher in Indica cultivars and wild species than in Japonica cultivars (Table 1.6) (Oka, 1988). Cross pollination between wild species and *O. sativa* cultivars has been reported to occur in natural habitats (Oka and Chang, 1961).

### B. Asexual reproduction

*O. sativa* is cultivated annually. However, rice plants can grow vegetatively and continuously under favourable water and temperature conditions, even after they have borne the seeds. This perennial character in *O. sativa* is considered to have been inherited from the ancestral species *O. rufipogon* (Morishima *et al.*, 1963).

Under natural conditions, tiller buds on the basal nodes of rice plants start to re-grow after rice grains have been harvested. These new tillers, called the “ratoon”, grow best under long-day conditions. In some countries, farmers grow ratoon plants to obtain a second harvest of rice.

Cell/tissue culture techniques can be used to propagate calli and reproduce tissues or plants asexually under the appropriate cultural conditions. Haploid plants are easily obtained through anther culture. They become diploid spontaneously or when artificially treated with chemicals (Niizeki and Oono, 1968).

### C. Reproductive barriers

Viable hybrids between *O. sativa* and distantly related varieties or species are difficult to achieve. The postmating barriers are classified into four types, namely  $F_1$  inviability (crossing barrier),  $F_1$  weakness,  $F_1$  sterility and hybrid breakdown (Oka, 1988). All these phenomena have been found in cultivated rice and its wild relatives, although the  $F_1$  plants whose parents have the AA genome in common show no significant disturbances in meiotic chromosome pairing (Chu *et al.*, 1969).

In many cases, cross-sterility comes from failure in the development of young  $F_1$  zygotes, particularly the development of endosperm, after fertilisation takes place. The African perennial species *O. longistaminata* showed a stronger crossing barrier with *O. glaberrima* and *O. breviligulata*<sup>11</sup> than with *O. sativa* and *O. rufipogon*<sup>12</sup> (Chu *et al.*, 1969).

$F_1$  weakness is controlled by complementary dominant weakness genes (Chu and Oka, 1972) which disturb tissue differentiation or chlorophyll formation.  $F_1$  weakness is rare in crosses between *O. sativa* cultivars (Amemiya and Akemine, 1963). Among strains of *O. glaberrima* and *O. breviligulata*<sup>11</sup>, about one-fourth of the crosses examined showed  $F_1$  weakness (Chu and Oka 1972).  $F_1$  weakness was found also in crosses between *O. longistaminata* and *O. glaberrima* or *O. breviligulata*<sup>11</sup>, between the American form of *O. perennis*<sup>13</sup> and *O. breviligulata*<sup>11</sup>, and between the Asian and Oceanian forms of *O. perennis* complex<sup>14</sup> (Oka, 1988).

11. The species name *O. breviligulata* used by Oka and his co-researchers is *O. barthii* in Table 1.

12. The species name *O. rufipogon* may have included the annual species *O. nivara* and the perennial species *O. rufipogon* in Table 1.

13. The American form of *O. perennis* is *O. rufipogon* in Table 1 and is sometimes called *O. glumaepatula*.

14. The Asian and Oceanian forms of *O. perennis* complex are *O. rufipogon* and *O. nivara* (in Asia) and *O. meridionalis* (in Australia) in Table 1.

F<sub>1</sub> sterility is frequently found in crosses of cultivated rices and their wild relatives, in which the failure of development of male and female gametes is often observed due to chromosomal disturbance in meiotic pairing or genetic disorders. Cytoplasmic pollen sterility and its fertility-restoration are reported in many crosses (Virmani, 1994).

Partial sterility appears in F<sub>2</sub> plants from crosses between distantly related *O. sativa* cultivars. The sterility is controlled by a set of complementary recessive sterility genes. It seems that there are many sets of complementary or duplicate sterility genes among cultivated and wild species (Kitamura, 1962; Oka, 1964).

The weakness and sterility occurring in the F<sub>2</sub> and later inbred generations are referred to as hybrid breakdown. Hybrid breakdown is controlled by a set of complementary recessive weakness genes (Oka, 1957; Okuno, 1986). Genes for F<sub>2</sub> weakness seem to be distributed occasionally in cultivated and wild rice species.

## 6. Crosses

### A. Intraspecific crosses

Although *Oryza sativa* is basically self-pollinated, natural outcrossing can occur at a rate of up to 5% (Table 1.6) (Oka, 1988). When different cultivars of the same maturity group are planted side by side in a field or in adjacent fields, natural outcrossing can occur between these cultivars. Outcrossing can be avoided by allocating cultivars with sufficiently different maturity time to adjacent fields, or by separating cultivars with the same maturity time.

F<sub>1</sub> plants from crosses within the Indica or Japonica group generally show high fertility in pollen and seedset. Those from crosses between the two groups have lower pollen fertility and lower seedset, with some exceptions, but F<sub>1</sub> fertility is not a good criterion for classifying cultivars into Indica-Japonica groups (Oka, 1988; Pham, 1991).

Hybrid progenies from Indica-Japonica crosses might survive, overcoming various reproductive barriers which are due to genetical and physiological disorders controlled by genic and cytoplasmic factors. Hybridisation between distantly related cultivars of the same species sometimes produces more vigorous hybrid plants than the parental cultivars with more descendant seeds, and establishes new ecotypes which are genetically different from the original population. Artificially selected hybrid plants thus produced may serve an important role in building new cultivars over a long historical period.

### B. Interspecific crosses

*O. sativa* and *O. glaberrima* are often grown as mixtures of various proportions in West African rice fields (Chu *et al.*, 1969). The two species resemble each other, perhaps due to co-evolution, but natural hybrids between them are rare, even though experimental hybridisation is easy. The F<sub>1</sub> plants are highly pollen-sterile, but about one-third of the F<sub>1</sub> embryo sacs are normal and functional. Backcrosses can be made with the pollen of either parent. The gene loci that have been examined are identical in the two species (Sano, 1988). Most natural hybrids disappear due to several genetic and physiological disorders, leaving only a very low probability of gene transmission between the two species.

*O. rufipogon*,<sup>15</sup> the wild progenitor of *O. sativa*, can be crossed with *O. sativa* and sometimes produces hybrid swarms in the field. Their hybrids show no sterility (Oka, 1988). The variation between

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15. The species name *O. rufipogon* used by Oka and his co-researchers may have included the annual species *O. nivara* and the perennial species *O. rufipogon* in Table 1.

perennial and annual types is nearly continuous, and some intermediate perennial-annual populations are most likely to be the immediate progenitor of cultivated rice because they have a high genetic variability, a moderately high seed productivity, and tolerance for habitat disturbance (Sano *et al.*, 1980).

*O. glaberrima* and its wild progenitor *O. breviligulata*<sup>16</sup> produce fertile hybrids and natural hybrid swarms in the fields. They have an annual growth habit and resemble each other in most botanical characters (Oka, 1991).

The common wild rices are distributed throughout the humid tropics and comprise geographical races such as Asian (*O. nivara* and *O. rufipogon*), African (*O. barthii* and *O. longistaminata*), American (*O. glumaepatula*)<sup>17</sup> and Oceanian (*O. meridionalis*). All these species share the AA genome, but they are separated from one another by F<sub>1</sub> pollen sterility (Chu *et al.*, 1969). However, some *O. longistaminata* plants growing in rice fields produce the plants, which are self-compatible and crossable with *O. sativa* (Chu and Oka, 1970; Ghesquiere, 1985). These are probably the result of gene introgression from cultivars across the reproductive barriers.

The relatively high seed-sets (9-73%) can be obtained through the artificial hybridisation of *O. sativa* with these AA genome wild species (Sitch *et al.*, 1989). *O. nivara* and *O. rufipogon* have been used in crosses with *O. sativa*. The former gives resistance to grassy stunt virus and the latter donates cytoplasmic male sterility (Khush and Ling, 1974; Lin and Yuan, 1980).

Species with the BB, BBCC, CC, or CCDD genome are more crossable with *O. sativa* (0-30% seedset) than the more distantly related EE and FF genome species with *O. sativa* (0.2-3.8% seedset), but their hybrids are highly male and female sterile (Sitch, 1990). Artificial gene transfer has been achieved through a series of backcrosses in crosses between *O. sativa* and *O. officinalis* for brown planthopper (*Nilaparvata lugens*) and white-backed planthopper (*Sogatella furcifera*) resistance (Jena and Khush, 1990) and *O. minuta* for blast and bacterial blight resistance (Amante-Bordeos *et al.*, 1992). Artificial crosses between *O. sativa* and more distantly related species such as *O. ridleyi* and *O. meyeriana* have been also reported, but the successful rate of such distant crosses was very low (Katayama and Onizuka, 1979; Sitch *et al.*, 1989). Artificial hybridisation in distant crosses is feasible, but requires embryo rescue to obtain F<sub>1</sub> hybrids and first backcross progenies.

16. The species name *O. breviligulata* used by Oka and his co-researchers is *O. barthii* in Table 1.

17. *O. glumaepatula* is the American form of *O. rufipogon* in Table 1.

**Table 1.6 Outcrossing rates estimated in wild and cultivated rice species by different methods (Oka, 1988)**

Taxa/type	Origin	Method	No. of populations	Outcrossing (%)	Reference
<i>Asian perennis</i> <sup>18</sup>					
Perennial	Taiwan	Marker gene	1	30.7	Oka, 1956
	Thailand	Marker gene	1	44.0	Oka & Chang, 1961
	Thailand	Isozyme markers	1	50.6	Barbier, 1987
Intermediate	Thailand	Isozyme markers	1	55.9	Barbier, 1987
Perennial	India	Variance ratio	1	37.4	Oka & Chang, 1959
	Sri Lanka	Variance ratio	2	22.4-26.5	Sakai & Narise, 1959
Annual	India	Variance ratio	1	21.7	Oka & Chang, 1959
	India	Variance ratio	3	16.6-33.9	Sakai & Narise, 1960
	India	Marker gene	1	7.9	Roy, 1921
	Thailand	Isozyme markers	1	7.2	Barbier, 1987
	India	Variance ratio	2	17.3-20.6	Oka & Chang, 1959
<i>Breviligulata</i> <sup>19</sup>	Africa	Variance ratio	2	3.2-19.7	Morishima <i>et al.</i> , 1963
<i>Sativa</i>	India	Marker gene	34	0-6.8	Butany (1957)
Indica	Africa	Marker gene	2	0-1.1	Roberts <i>et al.</i> , 1961
	Taiwan	Marker gene	4	0.1-0.3	Oka (unpubl.)
	Sri Lanka	Variance ratio	1	3.6	Sakai & Narise, 1960
Japonica	Taiwan	Marker gene	5	0.6-3.9	Oka (unpubl.)

## 7. Ecology

### A. Cultivation

In rice-growing environments, five water regimes are generally distinguished: irrigated, rain-fed shallow, deepwater, upland, and tidal wetland. Irrigated rice is dominant in Asia, while upland rice is dominant in Africa and Latin America. The proportion of rice culture types varies considerably country by country.

There are two types of rice culture: direct seeding and transplanting. In direct seeding cultivation, dry seeds or seeds that have been pre-soaked and pre-germinated are sown by hand or using seeding machines. With the transplanting method, young seedlings grown in nursery beds are transplanted by hand or transplanting machines to rice fields. In rice fields, plants start in the vegetative phase to make tillers, sheaths and leaves. Then the plants begin the reproductive phase, in which they make panicles and seeds. Seeds are harvested for food. Common diseases and pests are listed in Appendix II.

About 530 million tonnes of rice is harvested annually from plantings of 146 million hectares worldwide (FAO, 1995). More than 91% of world rice production comes from Asia, 5% from the

18 . The species name *O. perennis* used by Oka is *O. nivara* or *O. rufipogon* in the text.

19 . The species name *O. breviligulata* used by Oka is *O. barthii* in the text.

Americas, 3% from Africa, and another 1% from Europe and Oceania. Rice is used for food in various forms. Grains are heated in water to become cooked rice. Rice flour is usually kneaded with water, boiled and used for various rice products. The bran is an important source of oil for food and manufacturing. Husks are used for fertilisers and animal feed, and straw for making various materials for wrapping, mats, etc.

### **B. Volunteers and weediness**

Cultivars vary in the ease with which unhulled grains from panicles are shattered. This characteristic is influenced by the extent of the absciss layer between the hulls and the panicle rachis. Farmers have selected various cultivars, from easy to hard grain shattering, for hand and machine harvesting. Seeds shattered before or during harvesting are allowed to germinate, if the water and temperature regimes are favourable, and act as volunteer weeds both in paddy and upland fields where farmers might grow another cultivar of rice. In general, these shattered seeds and volunteer weeds will be buried or killed by normal agronomic practices such as plowing, drainage or flooding, and rotation. The Indica group has a wider range of grain shattering and greater potential to become a volunteer weed than does the Japonica group.

Seed dormancy enables seeds to remain viable from one season to the next. Non-dormant or weakly dormant seeds can germinate by themselves on the panicle, consequently losing their food grain value. Farmers and breeders have selected cultivars with the dormancy which is suited to the farming cycle. However, shattered seeds with dormancy will keep their longevity for several seasons and germinate sporadically in the fields when a new cultivar is planted. The factors related to seed dormancy exist in the hull, and dormancy enhances the ability of shattered seeds to become volunteer weeds. Indica has a wider range of seed dormancy than Japonica. Either Indica or Japonica red rice easily shatters and has strong dormancy, becoming a weed problem in rice fields. Intraspecific hybridisation between domesticated cultivars and their weedy relatives, including red rice, may occur in many rice-growing areas.

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## APPENDIX I

### Morphological and Genetic Characteristics of *Oryza* Species (after Vaughan, 1994 with additions from Aggarwal *et al.*, 1997)

#### *O. alta*

Tall (up to 4m), erect herb with broad leaves (generally >5cm), spikelets >7mm. Tetraploid (2n=48). CCDD genome. Latin and South America.

#### *O. australiensis*

Tall (>2m) erect herb, strap-shaped, gray green leaves, pear-shaped spikelets (6.5-9mm) with soft, wispy awn (<5cm) and scabrous panicle axis. Diploid (2n=24). EE genome. Australia.

#### *O. barthii*

Erect to semi-erect herb, leaves have short ligule (<13mm); spikelets, large (7.8-11mm), easily shattering, long, strong awns (up to 10cm) usually red; panicle rarely has secondary branching. Diploid (2n=24). AA genome. Africa.

#### *O. brachyantha*

Short (<1m), tufted, annual or weakly perennial, with slender culms; small, slender (<1.6mm wide) spikelets with long awns (6-17cm). Diploid (2n=24). FF genome. Africa.

#### *O. eichingeri*

Short (usually <1m) with hard, slender culms; glabrous ligule (<3.5mm); chlorophyllous veins the length of the immature spikelet; mature spikelets 4.5-6mm long. Diploid (2n=24). CC genome. Africa, Sri Lanka.

#### *O. glaberrima*

Great diversity of morphological characteristics, primary key characters are the lack of secondary and tertiary branching of the panicles, short (usually <10mm) and rounded ligule, spikelets generally awnless and non-shattering. Pubescence on leaves and spikelets usually sparse. Diploid (2n=24). West Africa.

#### *O. grandiglumis*

Tall (up to 4m) herb with broad leaves (3-5cm), pubescent ligule; sterile lemma the same length as palea and lemma. Tetraploid (2n=48). CCDD genome. South America.

#### *O. granulata*

Short (usually <1m) herb, lanceolate, dark green leaves; spikelets <6.4mm, always awnless, with granulate texture to palea and lemma. Tetraploid (2n=48). GG genome. Asia.

***O. latifolia***

Short (usually <1m) and tall (2m or more) forms exist. Leaves broad but <5cm; spikelet <7mm. Tetraploid (2n=48). CCDD genome. Latin and South America.

***O. longiglumis***

Erect to semi-erect tufted herb, usually 1-2m tall; spikelets 7-8mm long and 1.8-2.2mm wide, with trichomes in rows down the length of chartaceous (papery) palea and lemma; sterile lemma narrow and flexuous, as long or longer than fertile lemma; awn about 1cm long. Tetraploid (2n=48). HHJJ genome. New Guinea.

***O. longistaminata***

Tall (usually 2m or more), erect, rhizomatous herb, ligule of lower leaves >15mm, acute or 2-cleft; spikelets with anthers >3mm. Diploid (2n=24). AA genome. Africa.

***O. meridionalis***

Erect herb usually 1-2m tall; panicle branches tightly adpressed to main panicle axis, rarely having secondary branching; spikelets <2.3mm wide; awns 7.8-10.3cm. Diploid (2n=24). AA genome. Australia.

***O. meyeriana***

Short (usually <1m) herb, lanceolate, dark green leaves; spikelets >6.4mm long, awnless, with granulate texture to palea and lemma. Diploid (2n=24). GG genome. Asia.

***O. minuta***

Scrambling, stoloniferous herb; basal panicle branches usually not whorled; spikelets <4.7mm long and <2.0mm wide. Tetraploid (2n=48). BBCC genome. Philippines, New Guinea.

***O. nivara***

Short or intermediate height (usually <2m) herb; spikelets large, 6-8.4mm long, 1.9-3.0mm wide, 1.2-2.0mm thick; long, strong awn (4-10cm). Diploid (2n=24). AA genome. Asia.

***O. officinalis***

Erect, usually rhizomatous herb of variable height; basal panicle branches whorled with spikelets inserted half-way or more from base; spikelets <5.4mm long and >2.0mm wide. Diploid (2n=24). CC genome. Asia, New Guinea.

***O. punctata***

Erect herb of two morphological types, which correspond to two cytological types. Both morphological types have ligule >3mm, which is soft and splits when dried; basal panicle branches widely spreading; spikelets of diploid race >5.5mm long and <2.3mm wide, those of tetraploid race <5.5mm long and >2.3mm wide; awns of both races usually >3cm. Diploid (2n=24) and tetraploid (2n=48). BB and BBCC genome. Africa.

***O. rhizomatis***

Erect, rhizomatous herb, 1-3m tall; panicle without whorled basal panicle branches; spikelets inserted near base of lowest panicle branch; spikelet length >6mm with extenuated apiculus, often awnless. Diploid (2n=24). CC genome. Sri Lanka.

***O. ridleyi***

Erect to semi-erect tufted herb, usually 1-2m tall; spikelet 7.6-12.7mm long by 1.6-2.9mm wide, with rows of trichomes down the length of the chartaceous (papery) palea and lemma; sterile lemma narrow and flexuous, shorter than palea and lemma; awn about 1cm long. Tetraploid (2n=48). HHJJ genome. Asia, New Guinea.

***O. rufipogon***

Tufted and scrambling herb with nodal tillering; spikelets usually 8-9mm long but up to 11mm in Latin American race; anther usually >3mm, reaching 7mm or more; awn usually 6-10cm long but up to 16cm in Latin American race. Diploid (2n=24). AA genome. Asia, New Guinea, Australia, Latin and South America.

***O. sativa***

Great diversity of forms. Varietal diversity can be categorized into three major groups of the traditional varieties: (1) Indica varieties with usually slender, awnless grains, light green leaves, many tillers; (2) temperate Japonica varieties with usually roundish pubescent grains, dark green leaves, few tillers; (3) tropical Japonicas (Javanicas) usually large, rounded, awned, pubescent spikelets; low shattering; few tillers. Morphological criteria alone are insufficient to distinguish varietal groups. Diploid (2n=24). AA genome. Worldwide.

***O. schlechteri***

Short (50cm or less), stoloniferous herb with pubescent nodes; short, narrow leaves with pubescent auricle and short ligule; panicle short (<7cm) and spreading; spikelets <2mm long, awnless. Tetraploid (2n=48). Genome unknown. New Guinea.

## APPENDIX II

Common Diseases and Pests in *Oryza sativa*

## Virus diseases, mycoplasma-like organism diseases

Disease	Vector
Dwarf	<i>Nephotettix cincticeps</i> Uhler <i>Recilia dorsalis</i> Motschulsky
Black streaked dwarf	<i>Laodelphax striatellus</i> Fallen <i>Unkanodes sapporonus</i> Matsumura <i>Ribautodelphax albifascia</i> Matsumura
Grassy stunt	<i>Nilaparvata lugens</i> Stal.
Hoja blanca	<i>Sogatodes oryzicola</i> Muir <i>Sogatodes cubanus</i> Crawford
Orange leaf	<i>Recilia dorsalis</i> Motschulsky
Rugged stunt	<i>Nilaparvata lugens</i> Stal.
Stripe	<i>Laodelphax striatellus</i> Fallen <i>Unkanodes sapporonus</i> Matsumura <i>Ribautodelphax albifascia</i> Matsumura
Transitory yellowing	<i>Nephotettix apicalis</i> Motschulsky <i>Nephotettix cincticeps</i> Uhler <i>Nephotettix impicticeps</i> Ishihara
Tungro	<i>Nephotettix impicticeps</i> Ishihara <i>Nephotettix apicalis</i> Motschulsky <i>Nephotettix virescens</i> Distant <i>Nephotettix nigropictus</i> Stal. <i>Nephotettix parvus</i> Ishihara et Kawase <i>Nephotettix malayanus</i> Ishihara et Kawase
Yellow dwarf	<i>Recilia dorsalis</i> Motschulsky <i>Nephotettix virescens</i> Distant <i>Nephotettix cincticeps</i> Uhler <i>Nephotettix impicticeps</i> Ishihara <i>Nephotettix apicalis</i> Motschulsky <i>Nephotettix nigropictus</i> Stal.
Yellow mottle	<i>Sessilia pusilla</i> Gerstaecker

## Bacterial diseases

Disease	Agent
Bacterial blight	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> (Ishiyama) Swings <i>et al.</i> = <i>Xanthomonas campestris</i> pv. <i>oryzae</i> (Ishiyama) Dye
Bacterial leaf streak	<i>Xanthomonas oryzae</i> pv. <i>oryzicola</i> (Ishiyama) Swings <i>et al.</i>
Foot rot	<i>Erwinia chrysanthemi</i> Burkholder <i>et al.</i>
Grain rot	<i>Pseudomonas glumae</i> Kurita & Tabei
Pecky rice (kernel spotting)	Damage by many bacteria and fungi Feeding injury by rice stink bug
Sheath brown rot	<i>Pseudomonas fuscovaginae</i> (ex Tanii <i>et al.</i> ) Miyajima <i>et al.</i>

## Fungal diseases

Disease	Agent
Aggregate sheath spot	<i>Ceratobasidium oryzae-sativae</i> Gunnell & Webster (amorph: <i>Rhizoctonia oryzae-sativae</i> (Sawada) Mordue)
Bakanae disease	<i>Gibberella fujikuroi</i> (Sawada) Ito <i>Fusarium moniliforme</i> Sheldon
Black kernel	<i>Curvularia lunata</i> (Wakk.) Boedijin (teleomorph: <i>Cochliobolus lunatus</i> R.R. Nelson & Haasis)
Blast (leaf, neck, nodal and collar)	<i>Pyricularia oryzae</i> Cavara = <i>Pyricularia grisea</i> Sacc. (teleomorph: <i>Magnaporthe grisea</i> (Hebert) Barr)
Brown spot	<i>Cochliobolus miyabeanus</i> (Ito & Kuribayashi) Dreschler ex Dastur (anamorph: <i>Bipolaris oryzae</i> (Breda de Haan) Shoemaker)
Crown sheath rot	<i>Gaeumannomyces graminis</i> (Sacc.) Arx & D.Olivier
Downy mildew	<i>Sclerophthora macrospora</i> (Sacc.) Thirumalachar <i>et al.</i> , Eyespot <i>Drechslera gigantea</i> (Heald & F.A.Wolf) Ito
False smut (green smut)	<i>Ustilaginoidea virens</i> (Cooke) Takahashi
Kernel smut	<i>Tilletia barclayana</i> (Bref.) Sacc. & Syd. in Sacc. = <i>Neovossia horrida</i> (Takah.) Padwick & A. Khan
Leaf smut	<i>Entyloma oryzae</i> Syd. & P. Syd.
Leaf scald	<i>Microdochium oryzae</i> (Hashioka & Yokogi) Samuels & I.D. Hallett = <i>Rhynchosporium oryzae</i> Hashioka & Yokogi
Narrow brown leaf spot	<i>Cercospora janseana</i> (Racib.) O. Const. = <i>Cercospora oryzae</i> Miyake (teleomorph: <i>Sphaerulina oryzina</i> K. Hara)

Pecky rice (kernel spotting)	Damage by many fungi, including <i>Cochliobolus miyabeanus</i> (Ito & Kuribayashi) Drechs. ex Dastur. <i>Curvularia</i> spp. <i>Fusarium</i> spp. <i>Microdochium oryzae</i> (Hashioka & Yokogi) Samuel & I.C. Halett <i>Sarocladium oryzae</i> (Sawada) W. Gams & D. Hawksworth and other fungi
Root rots	<i>Fusarium</i> spp. <i>Pythium</i> spp. <i>Pythium dissotocum</i> Drechs. <i>Pythium spinosum</i> Sawada
Seedling blight	<i>Cochliobolus miyabeanus</i> (Ito & Kuribayashi) Drechs. ex Dastur. <i>Curvularia</i> spp. <i>Fusarium</i> spp. <i>Rhizoctonia solani</i> Kuhn <i>Sclerotium rolfsii</i> Sacc. (teleomorph: <i>Athelia rolfsii</i> (Curzi) Tu & Kimbrough and other pathogenic fungi
Sheath blight	<i>Thanatephorus cucumeris</i> (A.B. Frank) Donk (anamorph: <i>Rhizoctonia solani</i> Kuhn)
Sheath rot	<i>Sarocladium oryzae</i> (Sawada) W. Gams & D. Hawksworth = <i>Acrocyllindrium oryzae</i> Sawada
Sheath spot	<i>Rhizoctonia oryzae</i> Ryker and Gooch
Stackburn (Alternaria leaf spot)	<i>Alternaria padwickii</i> (Ganguly) M.B. Ellis
Stem rot	<i>Magnaporthe salvinii</i> (Cattaneo) R. Krause & Webster (synanamorphs: <i>Sclerotium oryzae</i> Cattaneo <i>Nakataea sigmoidae</i> (Cavara) K. Hara)
Water-mold (seed-rot and seedling disease)	<i>Achlya conspicua</i> Coker <i>Achlya klebsiana</i> Pieters <i>Fusarium</i> spp. <i>Pythium</i> spp. <i>Pythium dissotocum</i> Drechs. <i>Pythium spinosum</i> Sawada

## Nematodes

Pest	Agent
Cyst nematode	<i>Heterodera oryzae</i> Luc & Briz.
Root-knot nematodes	<i>Meloidogyne incognita</i> var. <i>acrita</i> Chitwood
Root nematode	<i>Hirschmaniella oryzae</i> Luc & Goodey
Stem nematode	<i>Ditylenchus angustus</i> (Butler) Filipjev
White tip (crimp nematode)	<i>Aphelenchoides besseyi</i> Christie

## Soil pests

Pest	Agent
Mole cricket	<i>Gryllotalpa orientalis</i> (=africana) Burmeister
Root aphids	<i>Tetraneura nigriabdominalis</i> Sasaki
Root weevils	<i>Geocica lucifuga</i> Zehntner <i>Echinocnemus squameus</i> Billberg <i>Lissorhoptrus oryzophilus</i> Kuschel <i>Echinocnemus oryzae</i> Marshall <i>Hydronomidius molitor</i> Faust

## Pests at the vegetative stage

Pest	Agent
Armyworms and cutworms	<i>Mythimna</i> (=Pseudaletia=Leucania=Cirphis) separata (=impuncta) Walker <i>Spodoptera mauritia</i> Boisduval <i>Spodoptera</i> (=Prodenia) litura Fabricius
Black bugs	<i>Scotinophara coarctata</i> Fabricius <i>Scotinophara lurida</i> Burmeister
Caseworm	<i>Nymphula depunctalis</i> Guenee
Field crickets	<i>Hieroglyphus banian</i>
Gall midge	<i>Orseolia</i> (=Pachydiplosis) oryzae Wood-Mason
Grasshoppers	<i>Locusta migratoria manilensis</i> <i>Oxya japonica japonica</i>
Green hairy caterpillar	<i>Rivula atimeta</i> Swinhoe
Green semilooper	<i>Naranga aenescens</i> Moore
Hispa	<i>Dicladispa</i> (=Hispa) armigera Oliver
Leaf beetle	<i>Oulema</i> (=Lema) oryzae Kuwayama
Leafhoppers	<i>Cnaphalocrocis medinalis</i> Guenee <i>Marasmia</i> (=Susumia) exigua Butler <i>Marasmia patnalis</i> Bradley <i>Marasmia ruralis</i> Walker
Mealybug	<i>Brevinnia</i> (=Heterococcus=Ripersia) rehi (=oryzae) Lindinger
Seedling maggots	<i>Atherigona oryzae</i> Mallock <i>Atherigona exigua</i> Stein
Stem bores	
dark-headed stem borer	<i>Chilo</i> (=Chilotraea) polychrysus (=polychrysa) Meyrick
pink stem borer	<i>Sesamia inferens</i> Walker

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striped borer	<i>Chilo suppressalis</i> Walker
white stem borer	<i>Scirpophaga</i> (=Tryporyza=Schoenobius) <i>innotata</i> Walker
yellow stem borer	<i>Scirpophaga</i> (=Tryporyza=Schoenobius) <i>incertulas</i> Walker
Thrips	<i>Stenchaetothrips</i> (=Baliothrips=Thrips) <i>biformis</i> (=oryzae) Bagnall
Whorl maggots	<i>Hydrellia philippina</i> Ferino
	<i>Hydrellia sasakii</i> Yuasa & Ishitani
	<i>Hydrellia griseola</i> Fallen

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### Pests at the reproductive stage

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Pest	Agent
Brown planthopper	<i>Nilaparvata lugens</i> Stal
Greenhorned caterpillar	<i>Melanitis leda ismena</i> Cramer
Green leafhoppers	<i>Nephotettix nigropictus</i> (=apicalis) Stal
	<i>Nephotettix virescens</i> (=impicticeps) Distant
	<i>Nephotettix cincticeps</i> Uhler
	<i>Nephotettix malayanus</i> Ishihara & Kawase
Skippers	<i>Pelopidas mathius</i> Fabricius
	<i>Parnara guttata</i> Bremer & Grey
Smaller brown planthopper	<i>Laodelphax striatellus</i> Fallen
Whitebacked planthopper	<i>Sogatella furcifera</i> Harvath
White leafhopper	<i>Cofana</i> (=Tettigella=Cicadella) <i>spectra</i> Distant
Zigzag leafhopper	<i>Recilia dorsalis</i> Motschulsky

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### Pests at the ripening stage

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Pest	Agent
Rice panicle mite	<i>Stenotarsonemus spinki</i> Smiley
Rice seed bugs	<i>Leptocorisa acuta</i> Thurnberg
	<i>Leptocorisa oratorius</i> Fabricius
	<i>Leptocorisa chinensis</i> Dallas

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### APPENDIX III

#### Transformation of Rice (*Oryza sativa*)

Rice breeding in rice-growing countries is supported by many breeding technologies that have been developed on the basis of long-accumulated research and experience gained with traditional practices. Major aspects of traditional rice breeding, including conventional practices and seed multiplication as well as early applications of biotechnology, such as anther culture and somatic mutation through protoplast and tissue culture - are well-described in another OECD publication (Kaneda, 1993)

More recent application of biotechnology to rice breeding, particularly genetic transformation of rice, was started in late 1980s. It was considered more difficult than in other plant species, as rice plant regeneration from protoplast requires special skills. The usual technique of using the Ti plasmid of *Agrobacterium tumefaciens*, which has been very effective for gene introduction in many dicot plant species, was not a useful tool for transferring foreign genes into rice. However, these problems have been overcome through recent progress in transformation techniques.

Currently, the following three methods of gene introduction are reported to have been used among researchers. There is one other method, using polyethyleneglycol (PEG), which has had only limited use in recent years.

The first is the electroporation method, which directly introduces foreign genes into protoplasts. Improvement in efficiency and stability of regeneration from protoplasts to plantlets is another factor contributing to the development of this method.

The second is the biolistic (particle gun bombardment) method, which directly introduces foreign genes into regenerable plant cells such as scutellum cells. The main merit of this method is that it eliminates the problems of regeneration from protoplasts and minimises the possibility of the occurrence of somaclonal variation during the regeneration process.

The third is the improved *Agrobacterium*-mediated method, which was initiated a few years ago. Its main merit includes insertion of a more precise gene construction, including promoters and marker genes on the plasmids, which results in improved efficiency of gene introduction as well as more stable expression and inheritance of the transgenes.

After the introduction of foreign genes into rice plant tissue, a suitable selection system is required to select plants that have been successfully transformed. In the case of rice, selection markers usually constitute genes that confer resistance to antibiotics. Among them, kanamycin was used in early stages, but most of the recent successful results of rice transformation have been obtained using hygromycin and geneticin (G418) because of their more efficient and stable function in selection procedures.

The types of traits expressed in transformed rice plants are similar to those expressed in the transformation of other plant species. This started with the introduction of marker genes in the early stages and expanded to include genes introduced so that some agronomically or industrially important traits could be expressed. The traits reported in recent successful transformations follow this trend, including pest and disease resistance, herbicide tolerance and specific grain quality.

Table AIII-1 summarises the information presented above in chronological order, to show the progressive development of rice transformation.

In this document, particularly Section IV (Identification Methods), recent progress in basic research on the rice genome is presented. It is expected that transformed rice plants with useful traits will be released for commercialisation in the near future, supported by developments both in basic genome research and in transformation technologies.

### Progress in the development of transformed rice

	Method of transformation	Introduced gene	Remarks
Junker <i>et al.</i> , (1987)	polyethyleneglycol	NPT-II	transient expression
Toriyama <i>et al.</i> , (1988)	electroporation	AMP-II	transformed plant
Shimamoto <i>et al.</i> , (1989)	electroporation	HPT	transformed plant
Battraw and Hall (1990)	electroporation	NPT-II, GUS	transformed plant
Hayashimoto <i>et al.</i> , (1990)	polyethyleneglycol	HPT	transformed plant
Raineri <i>et al.</i> , (1990)	<i>Agrobacterium</i>	NPT-II	callus formation
Christou <i>et al.</i> , (1991)	electroporation	<i>bar</i> , GUS	ransformed plant
Meijer <i>et al.</i> , (1991)	polyethyleneglycol	HPT, GUS	transformed plant
Murai <i>et al.</i> , (1991)	polyethyleneglycol	HPT, <i>Ac</i>	transformed plant
Battraw and Hall (1992)	electroporation	NPT-II, GUS	transformed plant
Cao <i>et al.</i> , (1992)	particle gun	<i>bar</i>	transformed plant
Datta <i>et al.</i> , (1992)	polyethyleneglycol	HPT, <i>bar</i>	transformed plant
Hayakawa <i>et al.</i> , (1992)	electroporation	HPT, CP of RSV	transformed plant
Li <i>et al.</i> , (1992a)	polyethyleneglycol	HPT, mutant ALS	transformed plant
Li <i>et al.</i> , (1992b)	polyethyleneglycol	HPT	transformed plant
Peng <i>et al.</i> , (1992)	polyethyleneglycol	NPT-II	callus formation
Chan <i>et al.</i> , (1993)	<i>Agrobacterium</i>	NPT-II, GUS	transformed plant
Fujimoto <i>et al.</i> , (1993)	electroporation	HPT, <i>cryIA(b)</i>	transformed plant
Shimamoto <i>et al.</i> , (1993)	electroporation	HPT, <i>Ac</i> transposon non-autonomous maize Ds element	transformed plant
Tada and Fujimura (1993)	electroporation	HPT antisense of allergen gene	transformed plant
Uchimiya <i>et al.</i> , (1993)	electroporation	<i>bar</i>	transformed plant
Wang <i>et al.</i> , (1993)	particle gun	GUS, CAT	transient expression
Hosoyama <i>et al.</i> , (1994)	electroporation	HPT, Oryzacystatin	transformed plant
Hiei <i>et al.</i> , (1994)	<i>Agrobacterium</i>	HPT, GUS	transformed plant
Xu and Li (1994)	electroporation	NPT-II	transformed plant
Zhu <i>et al.</i> , (1994)	lipofectin	NPT-II human $\alpha$ -interferon	transformed plant
Christou and Ford (1995)	particle gun	<i>bar</i> , GUS	transformed plant
Clough <i>et al.</i> , (1995)	particle gun	HPT oat phytochrome	transformed plant

Cooley <i>et al.</i> , (1995)	particle gun	a apoprotein	
Rashid <i>et al.</i> , (1995)	<i>Agrobacterium</i>	<i>bar</i> , GUS	transformed plant
Li and Murai (1995)	polyethyleneglycol	HPT, GUS	transformed plant
Lin <i>et al.</i> , (1995)	electroporation	HPT, <i>Ac</i>	transformed plant
Lynch <i>et al.</i> , (1995)	electroporation	HPT, chitinase	transformed plant
Peng <i>et al.</i> , (1995)	polyethyleneglycol	NPT-II	field trial
Duan <i>et al.</i> , (1996)	particle gun	NPT-II, GUS	transformed plant
Jain <i>et al.</i> , (1996)	particle gun	<i>bar</i> , pin2	transformed plant
Sivamani <i>et al.</i> , (1996)	particle gun	HPT, GUS, HVA-1	transformed plant
Xu <i>et al.</i> , (1996)	particle gun	HPT, GUS	transformed plant
Wunn <i>et al.</i> , (1996)	particle gun	<i>bar</i> , HVA-1	transformed plant
Zheng <i>et al.</i> , (1995)	polyethyleneglycol	HPT, <i>cryIA(b)</i>	transformed plant
Zhen <i>et al.</i> , (1996)	particle gun	HPT, $\alpha$ -phaseolin	transformed plant
Burkhardt <i>et al.</i> , (1997)	particle gun	HPT, GUS	transformed plant
		HPT	transformed plant
Nayak <i>et al.</i> , (1997)	particle gun	phytoene synthase	
Takano <i>et al.</i> , (1997)	polyethyleneglycol	HPT, <i>cryIA(c)</i>	transformed plant
Toki (1997)	<i>Agrobacterium</i>	HPT, Luc	transformed plant
Zheng <i>et al.</i> , (1998)	particle gun	HPT, <i>bar</i>	transformed plant
		NPT-II	transformed plant
		Eighth largest	
		segment of RDV	

NPT-II: neomycin phosphotransferase,  
 GUS:  $\beta$ -glucuronidase  
 CAT: chloramphenicol acetyltransferase  
 RSV: rice stripe virus  
*Ac*: maize transposable element Activator  
 ALS: acetolactate synthase  
 Luc: luciferase

HPT: hygromycin phosphotransferase  
 AMP-: aminoglycoside phosphotransferase  
*bar*: phosphinothricin acetyltransferase gene  
 RDV: rice dwarf virus  
 CP: coat protein  
 HVA-1: late embryogenesis abundant protein gene  
 pin2: potato proteinase inhibitor II (PINII) gene

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## SECTION 6

### POTATO (*Solanum tuberosum* subsp. *tuberosum*)

#### 1. General Information

This consensus document addresses the biology of the potato (*Solanum tuberosum* subsp. *tuberosum*). It contains general information on the taxonomy, morphology, and centre of diversity of the species which can be of importance during a risk assessment (for example, information on reproductive biology, the possibility of crosses, and ecology). In regard to intra- and interspecific crosses, emphasis has been given to the conditions which make a cross possible rather than listing all successful crosses. Such a list would be very long and subject to frequent changes. Only hybridisation events not requiring human intervention are considered.

The Netherlands served as lead country in the preparation of this document, in collaboration with the United Kingdom.

#### 2. Taxonomic Status

The family *Solanaceae* contains several well known cultivated crops such as tomato (*Lycopersicon esculentum*), eggplant (*Solanum melongena*), tobacco (*Nicotiana tabacum*), pepper (*Capsicum annuum*) and potato (*Solanum tuberosum*). The potato ranks, on a world scale, fourth in food production for starch crops. Around 300,000 metric tonnes are produced (FAO, 1985). About 95 countries produce potatoes, with a total value of around \$US 13 billion (Horton *et al.*, 1985).

Within the genus *Solanum* over a thousand species have been recognised. According to Burton (1989), there are "well over two thousand species". This genus is subdivided into several subsections, of which the subsection *potatoe* contains all tuber-bearing potatoes. The subsection *potatoe* is divided into series, one of which, *tuberosa*, is relevant to this document. Within the series *tuberosa* about 54 species, both wild and cultivated, are found. One of these is *S. tuberosum* (Hawkes, 1990).

*S. tuberosum* is divided into two subspecies: *tuberosum* and *andigena*. The subspecies *tuberosum* (Table 1.7) is the cultivated potato widely in use as a crop plant in, for example, North America and Europe. The subspecies *andigena* is also a cultivated species, but cultivation is restricted to Central and South America (Hawkes, 1990; Hanneman, 1994).

Table 1.7 Taxonomic position of *S. Tuberosum* subsp. *Tuberosum*

Taxonomic rank	Latin name
family	<i>Solanaceae</i>
genus	<i>Solanum</i>
section	<i>petota</i>
subsection	<i>potatoe</i>
series	<i>tuberosa</i>
species	<i>Solanum tuberosum</i>
subspecies	<i>tuberosum</i>

### 3. Centre of Diversity

The centre of diversity for wild tuber-bearing potatoes (subsection *potatoe*) lies in Latin America, which is also considered the centre of origin. For the series *tuberosa* (to which *S. tuberosum* belongs) and most other series within the subsection *potatoe*, there are two centres of diversity. One is a long-stretching Andean area in Venezuela, Colombia, Ecuador, Peru, Bolivia and Argentina. The other is in central Mexico. The distribution area of these wild potatoes is much larger: from the southwestern United States to southern Argentina and Chile (Child, 1990; Hawkes, 1990).

Generally the cultivated *Solanum* species are also found within the centres of diversity for wild potatoes. The exception is the cultivated diploid form of *Solanum tuberosum* subsp. *tuberosum*, which is only found in a constricted area of southwestern Chile.

The cultivated tetraploid *Solanum tuberosum* subsp. *tuberosum*, as known in Europe and most other parts of the world, is considered to be a selection from a small introduction of *S. tuberosum* subsp. *andigena* potatoes from Colombia and Peru, and as such has a very narrow genetic basis. The arguments for this thesis are that plants of the original introductions into Europe are known to have been late flowering and tuberising, and that the morphological description of these potatoes matches the *andigena* type (Howard, 1970). Through selection, this introduction was adapted to the longer day lengths and different environmental conditions of Europe. Simmonds (1966) has shown that such transition can take place in a fairly short period of approximately ten years of selection. From Europe, this new type of potato has spread all over the world as a cultivated crop. An alternative theory is that, after the potato blight epidemic in Europe, new germplasm of *S. tuberosum* subsp. *tuberosum* originating from Chile (Hawkes, 1990) was introduced into Europe.

### 4. Identification Methods

#### A. Morphology and somaclonal variation

The subsection *potatoe* is distinguished from all other subsections within the genus *Solanum* by "true potatoes whose tubers are borne on underground stolons, which are true stems, not roots" (Hawkes, 1994).

The series *tuberosa* is characterised by "imparipinnate or simple leaves, forked peduncle, rotate to petagonal corolla and round berries" (Hawkes, 1990). The species *S. tuberosum* is characterised by "pedicel articulation placed in the middle third, short calyx lobes arranged regularly, leaves often slightly arched, leaflets always ovate to lanceolate, about twice as long as broad, tubers with well marked dormancy period" (Hawkes, 1990).

The differences between the two subspecies within *S. tuberosum* are very small, the greatest difference being the short day dependence of the subspecies *andigena*. The differences are set out in Table 1.8.

Table 1.8 Distinction between *S. tuberosum* subsp. *tuberosum* and subsp. *Andigena* (Hawkes, 1990)

Characteristic	<i>tuberosum</i>	<i>andigena</i>
leaves	less dissected	dissected
leaflets	wider	narrow
leaf angle	arched	acute
pedicel	thickened at the apex	not thickened at the apex
tuber formation	long or short days, mostly altitude neutral	short days, higher altitudes

The general description of the morphology of *S. tuberosum* subsp. *tuberosum* is as follows: Herbaceous perennial with weak stems that grow to a maximum of three feet, long pinnate leaves, ovate leaflets with smaller ones disposed along the midrib. The flowers are white, purple, pinkish, or bluish, in clusters, usually with a five-part corolla and exerted stamens with very short filaments. The fruits are yellowish or green, globose, and less than one inch in diameter. Some lack seeds, but others may contain several hundred. The fruits are inedible by humans due to the presence of toxins (Anonymous, 1996; Hawkes, 1990). Tubers are borne at the end of underground stolons. They are round to long oval. The flesh is generally white or cream to yellow, the skin colour light brownish to red. Tubers can contain high levels of solanine, a toxic alkaloid.

Potatoes are very easily regenerated with the use of *in vitro* tissue culture techniques. This form of vegetative propagation normally leads to genetically identical individuals, but considerable heterogeneity is common after tissue culture in which a callus stage is included. This variation is called somaclonal variation. *S. tuberosum* subsp. *tuberosum* is, like all potatoes, quite prone to this kind of variation (Cutter, 1992; Hawkes, 1990).

#### A. Molecular identification

It is also possible to distinguish between several *Solanum* species with the use of molecular techniques. Restriction Fragment Length Polymorphism (RFLP) of chloroplast DNA, using eight endonucleases (Hosaka *et al.*, 1984), showed that 33 tuberous *Solanum* species and hybrids and two *Lycopersicon* species could be distinguished. The four different *Solanum tuberosum* subsp. *tuberosum* accessions showed identical patterns.

RFLP analyses of genomic DNA can also lead to species identification within the genus *Solanum*. Debener *et al.*, (1990) showed with the use of 70 probe/enzyme combinations (probes from *S. tuberosum* subsp. *tuberosum*) that 38 accessions representing twelve *Solanum* species and one hybrid could be distinguished. The two accessions from *S. tuberosum* subsp. *tuberosum* were not identical. One of the two accessions was the "true" *S. tuberosum* subsp. *tuberosum* cultivar Bintje, the other was a breeding line in whose pedigree *S. andigena*, *S. demissum* and *S. acaule* were introgressed. It could also be shown that RFLP fingerprinting allows distinction not only between species but also between different cultivars or breeding lines (Weising *et al.*, 1992). The use of probes from other *Solanum* species, mostly repetitive sequences, also led to species and cultivar identification (Schweizer *et al.*, 1993). Also RAPD markers can be used for potato cultivar and clone fingerprinting (Powell *et al.*, 1991; Quiros *et al.*, 1993).

In addition, there are indications that the *Solanum* species can be distinguished with the use of the Amplified Fragment Length Polymorphism (AFLP) technique. One primer can generate up to 120 bands per sample, so that discrimination between *Solanum* species is very easy (Kardolus, in press).

It appears that the potato and tomato genomes are so preserved that probes from one can be used to identify the other (Gebhart *et al.*, 1991). This is especially important for the construction of a genetic map and the development of molecular markers.

#### 5. Genetic Characteristics: The Genome

The basic chromosome number in the genus *Solanum* is twelve. *S. tuberosum* subsp. *tuberosum* can be diploids ( $2n=24$ ) or tetraploids ( $4n=48$ ). The diploids are only found in Chile, while the tetraploids are the most commonly cultivated all over the world. How the tetraploidy originated is unclear. The cultivated *S. tuberosum* subsp. *tuberosum* can be either an autotetraploid (doubling of the chromosomes of a diploid species) or an allotetraploid (doubling of the chromosomes of a diploid hybrid between two related species) (Hawkes, 1990).

The phenomenon of unreduced gametes is common in *Solanum* species. In most *Solanum* species, next to the normal haploid gametes ( $n$ ), unreduced gametes ( $2n$ ) can be found, greatly extending the possible number of natural crosses (see section on crosses) (Hanneman, 1995). Watanabe *et al.*, (1991) reported that most of the 38 tuber-bearing *Solanum* species examined produced  $2n$  pollen. The frequency varied from 2 up to 10 per cent.

It is also fairly easy to produce dihaploids from cultivated tetraploid *S. tuberosum*. This can be done by pollinating with, for example, *S. phureja*, which leads to the formation of parthenogenetic diploid plants. Anther culture is also in use to produce dihaploids (Howard, 1970; Caligari, 1992). It has been shown that, where *S. phureja* is used to produce dihaploids, minor chromosomal fragments are found in these dihaploids originating from *S. phureja* (Clulow *et al.*, 1991).

The great value of these diploids is in breeding programmes: species that do not cross readily with the tetraploid potato can cross with a dihaploid (see section on crosses below). These dihaploids are often ovule fertile but pollen sterile.

## 6. Reproductive Biology

### A. Sexual reproduction

Diploid *S. tuberosum* and the other diploid species within the section *petota* are self-incompatible (Kirch *et al.*, 1989). This incompatibility is of a gametophytic, multi-allelic nature based on the occurrence of  $S$  alleles. In general these species are insect-pollinating, cross-breeding species

The cultivated tetraploid *S. tuberosum* subsp. *tuberosum* is self-compatible. The  $S$  alleles occur in this species, but somehow the incompatibility system is weakened. The mechanism behind this is not known. Plaisted (1980) has shown that under field conditions selfing is most likely for tetraploid *S. tuberosum*, with 80-100 per cent of the seeds formed due to selfing.

To facilitate cross-breeding and selfing, the appearance of insects is necessary. In particular, bumblebees (*e.g.* *Bombus funebris* in Peru and *B. impatiens* in the US) are good pollinators for potatoes (White, 1983). Pollen dispersal is mainly limited by the distance pollinating insects fly. Bumblebees and bees do not fly much further than three kilometres (Reheul, 1987). Normal honeybees (*Apis mellifera*) and *Bombus fervidus* are not pollinators of potato, as the flowers are without any nectar (Sanford and Hanneman, 1981). White (1983) carried out some experiments to determine the importance of pollination by wind for potatoes. Flowers were emasculated, and therefore of no interest to insects. The seedset on these flowers was assessed. No seeds were found, and therefore it was concluded that pollination by wind was of no importance.

Conner *et al.*, (1996) collected outcrossing data from several field experiments with genetically modified potatoes, performed in New Zealand, the United Kingdom and Sweden. In each study the outcrossing rate was reduced to 0 per cent where the receiving plants were separated by more than 20 metres from the genetically modified ones.

Although many *Solanum* species are fertile, it appears that a large number of the tetraploid cultivated *S. tuberosum* subsp. *tuberosum* cultivars have a reduced fertility (Ross, 1986). Most cultivars show a reduced pollen fertility or even pollen sterility. Amongst them are well known cultivars like Bintje and King Edward. Although reduced female fertility is not so common, it is noticed that a lot of cultivars flower less profusely than wild material. Another observable phenomenon is that flowers are dropped after pollination, so that no berries are found. The result is that on most *S. tuberosum* subsp. *tuberosum* cultivars few berries and seeds are formed.

Potato seeds cannot be disseminated by birds, but dissemination by small mammals is possible (Hawkes, 1988). Lawson (1983) showed that in Scotland potato seeds could be stored in the ground for up to ten years without losing viability. Love *et al.*, 1994 report that potato seeds can survive and germinate for periods of time in excess of seven years.

## **B. Asexual reproduction**

The potato can also multiply vegetatively. Tubers are formed under the ground. As the tuber is the product for which potatoes are cultivated, an extensive selection has taken place for good tuber production and quality. These tubers can remain viable for long periods of time as long as there is not a major frost period. The stolons on which the tubers are formed are generally not very long for *S. tuberosum* subsp. *tuberosum* cultivars. Stolons of wild tuber-bearing *Solanum* species are much longer (Hawkes, 1990).

## **7. Crosses**

### **A. Intraspecific crosses**

*Solanum tuberosum* subspecies *tuberosum* and *andigena* are fully cross-compatible (Plaisted, 1980). Hybrids can occur in nature. The incidence of this cross is not clear, as the morphological distinction between the two subspecies is very small. As both subspecies only occur in southern North America and some parts of South America, natural crosses are only likely to be found there.

### **B. Interspecific crosses: crosses within the subsection *potatoe* (Tuber-bearing Potatoes)**

The gene pool for potato is extremely large. Dale *et al.*, (1992) and Evenhuis *et al.*, (1991) state that it is likely that all crosses between the tuber-bearing potatoes within the section *petota* may be possible, although in some cases techniques will have to be applied to establish the crosses.

It appears that there are two groups within this section which are very difficult to cross:

- The diploid species in the series *morelliformia*, *bulbocastana*, *pinnatisecta*, *polyadenia*, *commersoniana*, *lignicaulia*, and *circaeifoli*.
- The diploid species in the other series.

The fertilisation of a diploid plant with normal haploid pollen in fact consists of two fertilisations. The pollen contains two (haploid) generative nuclei; one nuclei fertilises the egg cell, the other fertilises the embryo sac nucleus. The result is a diploid embryo with triploid endosperm.

Den Nijs and Peloquin (1977) reported the existence of a "triploid block" where a tetraploid plant was crossed with a diploid plant. This block is due to the imbalance between the endosperm (5x) and the embryo (3x). The endosperm is not formed, and this is followed by embryo abortion (Jacobsen and Rousselle, 1992).

Johnston *et al.*, (1980, 1982) also found that some species of the same ploidy level could not cross, whereas crosses between species of different ploidy levels were successful. They introduced the concept of the Endosperm Balance Number (EBN), which is a measure to express the "effective ploidy of a genome in the endosperm". To make the normal development of the endosperm after fertilisation possible, the maternal EBN must be twice the paternal EBN (2:1).

The EBN is independent of the ploidy level of the species, and its behaviour is additive. This means, for instance, that by doubling of the chromosome number the EBN also doubles.

Two situations can occur:

- The EBN of two species is the same: natural crosses are possible;
- The EBN of two species is not the same: natural crosses are not possible.

Where the EBN of two species is not the same, several natural or artificial mechanisms are available to circumvent the incompatibility.

Natural mechanisms:

- The occurrence of unreduced gametes makes it possible that species with a lower EBN can cross with species with a higher EBN. For example:

A plant with 4x (EBN=4) cannot cross with a 2x (EBN=2) plant, but if the 2x plant produces unreduced gametes the EBN of these gametes becomes 4, which makes the cross possible. The resulting plant is a tetraploid (4x) with an EBN of 4. It is important to notice that, due to the common occurrence of unreduced gametes in most *Solanum* species, crosses of this kind can occur in nature.

Artificial mechanisms:

- Production of dihaploids makes it possible that species with a higher EBN can cross with species with a lower EBN. For example:

A plant with 4x (EBN=4) cannot cross with a 2x (EBN=2) plant. After dihaploidisation of the 4x (EBN=4) plant a diploid plant with an effective EBN of 2 is formed. This plant can be pollinated by the 2x (EBN=2) plant. The resulting plant is a diploid (2x) with an EBN of 2. It is important to notice that crosses of this kind are not likely to occur in nature, due to the fact that dihaploids are only rarely formed in nature.

- Polyploidisation of plants makes it possible to cross plants with a lower EBN with plants with a higher EBN. For example:

A plant with 2x (EBN=2) cannot cross with a 4x (EBN=4) plant. After polyploidisation of the 2x (EBN=2) plant a tetraploid plant with an effective EBN of 4 is formed. This plant can cross with the 4x (EBN=4) plant. The resulting plant is a tetraploid (4x) with an EBN of 4. It is important to notice that crosses of this kind are not likely to occur in nature, due to the fact that spontaneous polyploidisation rarely occurs; nevertheless, it cannot be excluded.

Despite the EBN system, potatoes of different groups can be combined by somatic fusion *in vitro*. The application frequency of this method is increasing. Fusion products may be fertile, so somatic hybrids may serve as a bridge for combining incompatible genomes.

In Annex I the ploidy and EBN of the most common potato species within the section *petota* are given. These data can be used as an indication of the possibility of formation of hybrids of *S. tuberosum* subsp. *tuberosum* with these species in nature. It is likely that *Solanum tuberosum* subsp. *tuberosum* can cross readily with all *Solanum* species mentioned in Annex I with the same EBN (=4). Also, due to the occurrence of unreduced gametes, the crosses of *S. tuberosum* subsp. *tuberosum* with all *Solanum* species mentioned in Annex I with an EBN of 2 are possible.

To determine if a cross really is likely to occur in nature, several factors have to be considered. The most important are:

- The EBN of the crossing partners:

These must be matching, or the EBN of one partner must not be less than half the EBN of the other partner.

- Geographical occurrence of the species involved:

The species involved must occur in the same area and habitat.

- Flowering period of the species involved:

The flowering periods must overlap.

- The presence of stylar barriers that prevent the growth of pollen tubes:

The presence of appropriate pollinators.

In most parts of the world, no *Solanum* species from the section *petota* with an EBN of 2 or 4 will occur next to cultivated tetraploid *S. tuberosum* subsp. *tuberosum*. Crosses are therefore not likely, due to geographical isolation. Only in the southern United States and South America do crossing partners with a suitable EBN occur next to cultivated tetraploid *S. tuberosum* subsp. *tuberosum*. In these areas the characteristics of the species involved and the habitat must be assessed to predict the likelihood of the cross.

### C. Interspecific crosses: crosses with species from sections other than *petota*

The occurrence of hybrids with species from sections other than *petota* is not likely, due to strong crossing barriers, although in several areas of the world cultivated tetraploid *S. tuberosum* subsp. *tuberosum* occurs next to indigenous *Solanum* species (not from section *petota*). For instance, *S. nigrum* and *S. dulcamara* occur in the Netherlands. Eijlander and Stiekema (1990) found that the cross of tetraploid *S. tuberosum* subsp. *tuberosum* with *S. dulcamara* did not result in any viable seeds and plants. For the cross of *S. nigrum* with *S. tuberosum* the same is valid. Only after embryo rescue could two hybrids be obtained. These were less vital, male sterile, formed no tubers, and showed less female fertility. After pollination (backcross), no viable seeds were formed.

## 8. Ecology of *Solanum tuberosum* subsp. *tuberosum*

### A. Cultivation

Environmental conditions under which *S. tuberosum* can be successfully grown are very diverse, as can be concluded from the fact that potatoes are cultivated in many parts of the world. A broad spectrum of cultivars are adapted to these different environmental conditions. Some general parameters can be determined for the cultivation of *S. tuberosum* subsp. *tuberosum*:

- The *S. tuberosum* subsp. *tuberosum* tuber cannot survive a temperature of -3°C and lower. The foliage dies at temperatures of -4°C (van Swaaij *et al.*, 1987; Vayda, 1994). Dale (1992) reports that potato tubers are destroyed by a frost period of 25 hours at -2°C or a frost period of five hours at -10°C. Latin American *Solanum* species can be much more frost-resistant.

- *S. tuberosum* subsp. *tuberosum* cannot be acclimatised to lower temperatures, whereas other *Solanum* species (for example, *S. acaule*) can be (van Swaaij *et al.*, 1987; Li and Fennel, 1985).
- Potatoes seem to be very sensitive to soil water deficit (Vayda, 1994).
- A wide range of soil pH can be tolerated by potatoes (normally 5 and higher, but good production at pH 3.7 was observed) (Vayda, 1994).
- *S. tuberosum* subsp. *tuberosum* is a daylight neutral crop, which means that tubers are made at a growth stage independent of the day length. But variation for daylight sensitivity can be found among *S. tuberosum* subsp. *tuberosum* cultivars.
- Extreme low or high temperatures, in particular the night temperature, can obstruct tuber formation.
- Short days (-14 hours) and moderate ground temperatures (15-18°) enhance tuber formation. Longer days (14-16 hours) and higher (day) temperatures (20-25°) enhance flowering and seed formation (Beukema and van der Zaag, 1979; Burton, 1989).
- Germplasm from all over the world has been used to improve potato varieties. The main goal is to develop cultivars with resistance to biotic factors (fungal, virus, bacterial and insect resistance). Other goals are: improved starch content, adaptation to tropical growing conditions, herbicide resistance, stress tolerance, and the introduction of anti-bruise genes (Brown, 1995). The species most used to improve potato are *S. demissum*, *S. acaule*, *S. chacoense*, *S. spegazzinii*, *S. stoloniferum*, *S. vernei*. Less used are *S. microdontum*, *S. sparsipilum*, *S. verrucosum*, *S. phureja*, *S. tuberosum* subsp. *andigena*, *S. commersonii* and *S. maglia* (Caligari, 1992). This germplasm has been introduced into many cultivars of *S. tuberosum* subsp. *tuberosum*.

In Annex II the most common diseases (insects, mites, viruses, bacteria and fungi) in potato and their spread throughout the world are shown. This annex is not intended to give a complete list of all potato diseases known. Therefore it should be taken into account that locally other diseases can be of great importance. The national phytosanitary service can best be consulted on this subject. Moreover, Annex II does not give any indication of which sanitary or quarantine provisions have to be applied in a country.

## B. Volunteers and weediness

In the cultivation of *S. tuberosum* subsp. *tuberosum*, plants from seeds from a previous potato crop can act as a volunteer weed. The tubers can also act as a volunteer weed in cultivation. In general these plants (from seeds and tubers) will be eliminated by normal agronomical practices. In addition, tubers will not survive for a long time in most of the areas of cultivation due to unfavourable environmental conditions (low temperatures).

Outside the field, potato seedlings will have difficulty establishing themselves as they cannot compete with other plants. Love *et al.*, 1994 report that these seedlings are limited to cultivated areas for reasons of competition and adaptation. Potato tubers can be spread during transportation and use, but generally these plants will not be established for a long time due to unfavourable environmental conditions.

In general, the potato is not known as a coloniser of unmanaged ecosystems. In climax vegetation it is not able to compete with other species such as grasses, trees and shrubs (Anonymous, 1996).

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## APPENDIX 1

Ploidy and EBN of Most *Solanum* Species within the Section *petota*  
(Hawkes, 1990; 1992; 1994)

SUBJECT	SERIES	PLOIDY	EBN=1	EBN=2	EBN=4	EBN=unknown
<i>catenifera</i>		Diploid	all species			
<i>juglandifolia</i>		Diploid				all species
<i>potatoe</i>	<i>morelliformia</i>	Diploid				<i>S. morelliformia</i>
	<i>bulbocastana</i>	Diploid	<i>S. bulbocastanum</i>			<i>S. clarum</i>
		Triplod	<i>S. bulbocastanum</i>			
	<i>pinnatifidus</i>	Diploid	<i>S. branchiatotrichum</i> <i>S. janczii</i> <i>S. cardiophyllum</i> <i>S. pinnatifidum</i> <i>S. trifidum</i>			<i>S. nani</i>
		Triplod	<i>S. cardiophyllum</i>			<i>S. janczii</i>
	<i>polyadenia</i>	Diploid				<i>S. polyadenium</i> <i>S. latvii</i>
	<i>commerzoniana</i>	Diploid	<i>S. commerzonii</i>			
		Triplod	<i>S. commerzonii</i>			<i>S. calvescens</i>
	<i>circosifolia</i>	Diploid	<i>S. capricorniculatum</i> <i>S. circosifolium</i>			
	<i>lignicaulis</i>	Diploid	<i>S. lignicaulis</i>			
	<i>obtusata</i>	Diploid				<i>S. obtusata</i>
	<i>yangtzensia</i>	diploid		<i>S. charoense</i> <i>S. tarijense</i>		<i>S. araege</i> <i>S. yangtzensia</i>
	<i>megistacroloba</i>	diploid		<i>S. astleyi</i> <i>S. megistacrolobum</i> <i>S. sanctae-rosae</i> <i>S. toralapanum</i>		<i>S. bolivienne</i>
	<i>cuneolata</i>	diploid		<i>S. infundibuliforme</i>		

SUBJECT.	SERIES	PLOIDY	EBN=1	EBN=2	EBN=4	EBN=unknown
	<i>conicibaccata</i>	diploid		<i>S. chomatophillum</i> <i>S. violaceumarmorum</i> <i>S. agrimonifolium</i> <i>S. colombianum</i> <i>S. oxycarpum</i>		<i>S. santolalle</i>
		hexaploid			<i>S. moscopanum</i>	
	<i>piurana</i>	diploid				<i>S. piurac</i>
	<i>ingifolia</i>	diploid				<i>S. ingifolium</i>
	<i>maglia</i>	diploid				<i>S. maglia</i>
		triploid				<i>S. maglia</i>
	<i>tuberosa</i>	diploid		wild: <i>S. berthaultii</i> <i>S. brevicaule</i> <i>S. bukasovi</i> <i>S. canasense</i> <i>S. gourlayi</i> <i>S. horizianum</i> <i>S. lophophyes</i> <i>S. medians</i> <i>S. microdonum</i> <i>S. multilobatum</i> <i>S. multilobatum</i> <i>S. paripilum</i> <i>S. spegazzinii</i> <i>S. vernalis</i> <i>S. verrucosum</i> cultivated: <i>S. phureja</i> <i>S. stenosomum</i>		wild: <i>S. alandense</i> <i>S. hondurani</i> <i>S. neocardenasii</i> <i>S. okadae</i> <i>S. oplocense</i>  cultivated: <i>S. ajanhuiri</i>
		triploid				wild: <i>S. maglia</i> <i>S. microdonum</i> cultivated: <i>S. chaucha</i> <i>S. xperczukii</i>
		tetraploid			wild: <i>S. gourlayi</i> <i>S. oplocense</i> <i>S. sucretae</i> cultivated: <i>S. tuberosum subsp. tuberosum</i> <i>S. tuberosum subsp. andigena</i>	
		pentaploid				<i>S. xcartilobum</i>
		hexaploid			<i>S. oplocense</i>	

Part 1 – Consensus Documents on Biology of Crops

SUBSECT.	SERIES	PLOIDY	EBN=1	EBN=2	EBN=4	EBN=unknown
	<i>amabilis</i>	tetraploid		<i>S. acule</i>		
		hexaploid			<i>S. albicans</i>	
	<i>longipedicellata</i>	triploid				<i>S. xavallu-mexici</i>
		tetraploid		<i>S. fendleri</i> <i>S. hyeritensis</i> <i>S. papita</i> <i>S. polytrichos</i> <i>S. moloniiformis</i>		
SUBSECT.	SERIES	PLOIDY	EBN=1	EBN=2	EBN=4	EBN=unknown
	<i>demissa</i>	triploid				<i>S. xsonidomissum</i>
		hexaploid			<i>S. brachycarpum</i> <i>S. demissum</i> <i>S. guerrerensis</i> <i>S. longasii</i> <i>S. topetalum</i>	<i>S. scheeki</i>

## APPENDIX II

The most common diseases in *Solanum tuberosum* subsp. *tuberosum* and their distribution: for each pest/disease category a reference to a more extensive review is given (Hilde and Lapwood, 1992; Evans and Trudgill, 1992; Raman and Radcliffe, 1992)

<b>INSECT AND MITE PESTS (review: Hooker, 1986)</b>	
<b>Organism</b>	<b>Occurrence</b>
<i>Myzus persicae</i> , <i>Phthorimaea operculella</i> , <i>Agriotes</i> spp.	Worldwide
<i>Macrosiphum euphorbiae</i> , <i>Aphis fabae</i> , <i>Empoasca devastans</i> , <i>Heliothus armigera</i> , <i>Spodoptera exigua</i>	Worldwide except Africa
<i>Plusia orichalcea</i> , <i>Sthenaridea pulsilla</i> , <i>Psylloides plana</i> , <i>Epicauta hirticornis</i> , <i>Anomala dimidiata</i> , <i>Phyllognathus dionysius</i> , <i>Melolontha</i> spp., <i>Odontotermes obesus</i> , <i>Eremotermes</i> spp., <i>Alicododes westermanni</i> , <i>Myllocerus subfasciatus</i> , <i>Pyralis farinalis</i> , <i>Nipaeococcus vastator</i>	Asia
<i>Empoasca fabae</i> , <i>Paratrioza cockerelli</i> , <i>Hypolithus</i> spp.	North America
<i>Diabrotica</i> sp., <i>Epicauta</i> spp., <i>Premnotypes</i> spp., <i>Phylophaga</i> spp., <i>Scrobipalpula absoluta</i> , <i>Scrobipallopis solanivora</i> , <i>Symmetrischema plaeoseosema</i> , <i>Feltia experta</i> , <i>Stenotycha</i> spp., <i>Copitarsia turbata</i> , <i>Benthinus mainen</i> , <i>Phenacoccus grenadensis</i> , <i>Liriomyza</i> spp.	Central and South America
<i>Shistocerca gregaria</i> , <i>Liriomyza trifolii</i>	Africa
<i>Henosepilachna sparsa</i> , <i>H. vigintisexspunctata</i> , <i>Austroasca virigrisea</i> , <i>Listroderes obliquus</i> , <i>Heteronychus arator</i> , <i>Cheiroplatys latipes</i> , <i>Graphognathus leucoloma</i>	Australia
<i>Aphis nasturtii</i> , <i>Limoniuss</i> spp., <i>Ctenicera</i> spp., <i>Conodorus</i> spp.	North America and Europe
<i>Aphis gossypii</i>	Central and South America, Asia
<i>Aulacorthum solani</i>	North America, Europe and Africa
<i>Leptinotarsa decemlineata</i>	North America, Europe and Asia
<i>Epitrix</i> spp.	North, Central and South America
<i>Epilachna</i> spp., <i>Polyphagotarsonemus latus</i> , <i>Thrips palmi</i> , <i>Gryllotalpa africana</i>	Africa and Asia
<b>NEMATODES (review: Hooker, 1986)</b>	
<b>Organism</b>	<b>Occurrence</b>
<i>Globodera rostochiensis</i> , <i>Globodera pallida</i>	Worldwide
<i>Meloidogyne hapla</i>	North America and temperate areas in general
<i>Meloidogyne chitwoodi</i>	Northwest America and parts of Western Europe
<i>Nacobbus aberrans</i>	Peru and Bolivia
<i>Pratylenchus penetrans</i> , <i>Trichodorus</i> and <i>Paratrichodorus</i> spp.	North America and Europe

<i>Dirtylenchus destructor</i>	North America, Western Europe and the former USSR
<i>Dirtylenchus dipsaci</i>	Western Europe
<b>VIRUSES (review: Valkonen, 1994)</b>	
<b>Organism</b>	<b>Occurrence</b>
Virus X (mild mosaic), leaf roll virus, Virus Y (severe mosaic), Virus A (mild mosaic), Virus S	Worldwide
Virus M	Europe and North America
Tobacco rattle virus	Europe, North America, Brazil and Japan
Mop top virus	Western Europe and Peru
Yellow dwarf virus	North America
Spindle tuber "viroid"	North America, former USSR and South Africa
Witches' broom (mycoplasma)	Europe, North America, Australia, China
<b>BACTERIA (review: Hooker, 1986)</b>	
<b>Organism</b>	<b>Occurrence</b>
<i>Clavibacter michiganensis</i> var. <i>sepedonicus</i> (ring rot), <i>Erwinia carotovora</i> ssp. <i>atroseptica</i> and subsp. <i>Carotovora</i> (blackleg, soft rot)	Europe and North America
<i>Erwinia chrysanthemi</i> (blackleg, soft rot)	Tropics and sub-tropics
<i>Streptomyces scabies</i> (common scab)	Worldwide
<i>Pseudomonas solanacearum</i> (brown rot)	Tropics and warm temperate zones
<b>FUNGI (review: Hooker, 1986)</b>	
<b>Organism</b>	<b>Occurrence</b>
<i>Alternaria solani</i> (early blight), <i>Botrytis cinerea</i> (grey mould), <i>Collectotrichum coccodes</i> (black dot), <i>Helicobasidium purpureum</i> (violet root rot), <i>Phytophthora infestans</i> (late blight), <i>Pythium ultimum</i> (watery wound rot), <i>Rhizoctonia solani</i> (black scurf), <i>Spongopora subterranea</i> (powdery scab), <i>Verticillium albo-atrum</i> , <i>V. dahliae</i> (wilt)	Worldwide
<i>Angiosorus solani</i> (potato smut)	Central and South America
<i>Fusarium</i> spp. (wilt)	North America
<i>Fusarium solani</i> var. <i>Coeruleum</i> (dry rot), <i>Phoma foveata</i> (gangrene)	Europe
<i>Helminthosporium solani</i> (silver scurf), <i>Sclerotinia sclerotiorum</i> (stalk break)	Europe and North America
<i>Macrophomina phaseolina</i> (charcoal rot)	North America and India
<i>Polyscytium pustulans</i> (skin rot)	Northern Europe, North America, Australia
<i>Synchytrium endobioticum</i> (wart)	Europe, North and South America, South Africa and Asia
<i>Phoma exigua</i> var. <i>Exigua</i> (gangrene), <i>Phytophthora erythroseptica</i> (pink rot)	Europe, North America and Australia

## SECTION 7

### BREAD WHEAT (*TRITICUM AESTIVUM*)

#### 1. General Description and Use as a Crop, Including Taxonomy and Morphology

*Triticum aestivum*, bread wheat, belongs to the order *Poales* (*Glumiflorae*), family *Poaceae* (*Gramineae*), tribe *Triticeae*, genus *Triticum*. The tribe *Triticeae* consists of 18 genera which are divided into two sub-groups, the *Triticinae* and the *Hordeinae*. The major genera in the sub-group *Triticinae* are *Triticum*, *Aegilops*, *Secale*, *Agropyron* and *Haynaldia* (Odenbach 1985, Zeller 1985, Körber-Grohne 1988).

Plants of the genus *Triticum* are annuals with spring or winter forms. They show the following morphological features: short ligule and spikelets that are sometimes hairy, and a smooth, bald, usually hollow culm, 0.7-1.6 metre in height. Pithy filling is less common than a hollow culm. The ears have a brittle or tough rachis. Generally they are four-sided. The spikelets have two to five florets. Each floret can produce one grain (caryopsis), i.e. is distichous. The glumes are keeled, on the upper side for example in *T. aestivum*, with serrated lemmas, long and either bearded or unbearded. Grains are loosely enclosed (naked wheat) and easily threshed. The rachilla has thin walls and does not disarticulate on maturity. In case of *T. aestivum* ssp. *spelta* (spelt wheat) the grains are hulled by the *spelta*. For this reason they cannot be dropped during the process of threshing (Garcke 1972, Geisler 1991).

*T. aestivum* is a cereal of temperate climates. The northern limit of wheat cultivation in Europe lies in southern Scotland (60° latitude) and occasionally beyond (central Scandinavia up to 64°). In North America wheat is grown to about 55° latitude. Wheat occurrence follows a similar pattern in the southern hemisphere. In the Alps, it is grown to an altitude of 1 500 metres above sea level (Körber-Grohne 1988, Geisler 1991).

The minimum temperature for germination of *T. aestivum* seeds is between 3 and 4°C. Flowering begins above 14°C. The vegetative period is 120 to 145 days for spring wheat and 280 to 350 days for winter wheat. Some varieties of *T. aestivum* need long photoperiods; some, especially those cultivated in southern Europe, are insensitive to day length. The harvested fruit, a grain with the botanical name caryopsis, contains approximately 80 to 84 per cent endosperm, approximately 60 per cent carbohydrate (starch), approximately 10 to 16 per cent protein, approximately 2 per cent fat, and approximately 13 per cent water (Hömmö and Pulli 1993). The starch granules of the *Triticeae* are botanically distinctive. Wheat meal is an important product. Meal from *T. durum* (macaroni wheat), for example, is used for the production of pastas such as spaghetti and semolina. Meal from *T. aestivum* (bread wheat) on the other hand contains a high proportion of gluten. For this reason it is very suitable for baking. Spelt wheat is rich in protein. Overlapping in protein content and high starch content can occur, as there is a wide range of difference due to both genetic variation and variable environmental conditions (Körber-Grohne 1988).

## 2. Agronomic Practices

In the Northern Hemisphere, depending on the location and the preceding crop, winter wheat can be sown from late August to late December. Sowing usually occurs between mid-September and late October. Seeds of winter wheat need 40 to 70 days vernalisation with a temperature between -1°C and +8°C (Geisler 1970, 1971, Kübler 1994). Hömmö and Pulli (1993) reported a maximum cold tolerance for winter wheat of about -25°C.

Seeds of spring wheat need only 3 to 5 days (Geisler 1970) or 0 to 14 days (Reiner *et al.*, 1992) vernalisation. The commencement of growth of shoots is decisively influenced by the photoperiod in the case of spring wheat. The cold tolerance for seedlings of spring wheat is about -5°C (Hömmö and Pulli 1993). The sowing season for spring wheat is from January to May (Kübler 1994).

In normal agricultural practice *T. aestivum* is used in a crop rotation schedule. Sugar beet, grain legumes and corn (*Zea mays*) or fodder maize make good preceding crops (Kübler 1994). Oilseed rape and winter barley occupy large areas and are part of many crop rotation systems that include winter wheat. Wheat/fallow rotations are commonly used in the western Great Plains region of the United States. Problems with plant diseases (see Annex I) may arise from the frequent use of wheat as part of the crop rotation system.

As with all crops cultivated and harvested at the field scale, some seeds may escape and remain in the soil until the following season when they germinate either before or following seeding of the succeeding crop. In some instances these “volunteers” may give considerable competition to the seeded crop and warrant chemical and/or mechanical control. The problem of volunteer plants in succeeding crops is common to most field crop species. Much depends on the management practices used in the production of the crop, *e.g.* the speed of the harvesting operation which will determine whether more or less seed is lost by the harvester. A suitable soil treatment after the harvest can considerably reduce the volunteer problem.

A great number of dicotyledonous and fewer monocotyledonous weeds have been reported to occur in fields used for wheat production. Seeds of some of these, when harvested and mixed with the wheat grain, can reduce flour quality (Wolff 1987).

Isolation of wheat plants for seed multiplication within the context of plant breeding can be done with greaseproof paper or cellophane bags placed over the heads (Mandy 1970, Saatgutverordnung/BGBl 1986). Without these, modest spatial isolation may be required to prevent outcrossing. In Germany, for example, there is no minimum isolation distance for wheat breeding, but there is a requirement for separation from all neighbouring plants that can be threshed, and for a buffer zone of a minimum of 40 cm to prevent mechanical mixing of the seeds (Saatgutverordnung 1986).

## 3. Centres of Origin/Diversity, Geographic Distribution

### A. History of wheat

The oldest archaeological findings of naked wheat (6800 to 5200 B.C.) come from southern Turkey, Israel, Syria, Iraq, Iran and south of the Caucasus Mountains in Georgia. At that time, einkorn, emmer and barley were the staple cereal crops in Asia Minor. Wheat was only grown on a regional basis. There is evidence that naked wheat was cultivated in the southern Caucasus in neolithic settlements between the late fifth and early fourth millennium B.C. Late Bronze Age specimens (approximately 1000 to 900 B.C.) of naked wheat have been found at several sites in the Crimea,

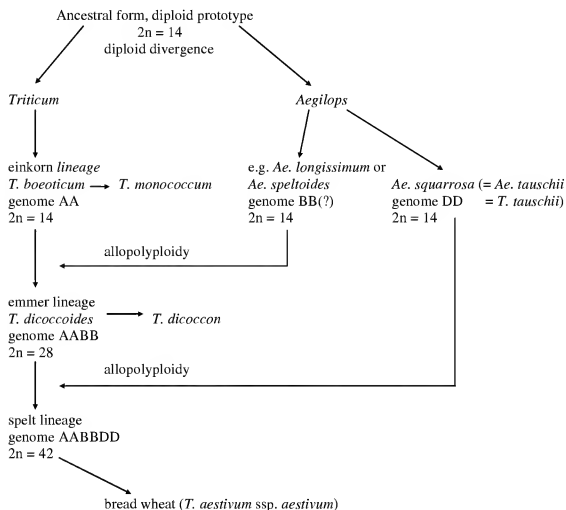
which was an early and significant wheat-growing area. Archaeological findings of wheat in Israel date from the same period (Körber-Grohne 1988).

In Central Europe, the oldest dated findings of wheat grains (a mixture of *T. aestivum*, *T. dicoccon* and *T. monococcum*) were in soil samples from the New Stone Age (4600 to 3800 B.C.). When the late neolithic period began, naked wheat was gaining importance as a crop in some areas along the River Neckar and around riverside and moorland settlements in the northern foothills of the Alps. It was not until the Roman Empire that wheat spread to the lower Rhine regions, the lower Meuse and the Scheldt Estuary, where it became the main cereal crop. Further south, spelt was favoured. Wheat farming declined north of the Alps between the fall of Rome and the Middle Ages. Evidence from excavated sites shows that little wheat was grown in the period 800 to 1200 (Körber-Grohne 1988).

The origin of Wheat has been well known since the 1940s, mainly through the work of E. R. Sears at the University of Missouri, Columbia (USA) from 1939 to 1980 (MacFadden and Sears 1946). The evolution of wheat began with an unknown diploid prototype, from which the genera *Triticum* and *Aegilops* were formed by diploid divergence. The development of the genus *Triticum* (see Figure 1.4) began with the einkorn lineage (*T. monococcum* line, genome AA), which developed into the cultured form *T. monococcum* from the wild form *T. boeoticum*. Allopolyploidization with an *Ae. speltoide*s descendant (genome BB) led to the tetraploid emmer lineage (*T. turgidum* line, genome AABB) with the wild form *T. dicoccoides* from which the cultured form *T. dicoccon* developed. The origin of the B-genome is more uncertain; *Ae. speltoide*s, *Ae. longissimum*, *Ae. bicornis*, *Ae. searsii*, *Ae. sharonense* are suggested as possible progenitors. The spelt lineage<sup>20</sup> with the genome AABBDD resulted from further allopolyploidization with the species *Ae. squarrosa* (= *Ae. tauschii*; genome DD) (Körber-Grohne 1988, Sitte *et al.*, 1991, Zeller and Friebe 1991). For the current classification of the genus *Triticum* see the monograph of van Slageren (1994), also available on the home page of the Wheat Genetics Research Center, Kansas State University (<http://www.ksu.edu/wgrc>, under “Triticum” accessions). More recent references in regard to the issue of wheat origin are Caudeyron (1994), Zohary and Hopf (1994) and Feldman *et al.*, (1995).

20. Note that the term “lineage” is used to indicate that descendants are related.

**Figure 1.4 An overview of the diploid einkorn lineage. (Körber-Grohne, 1988, Sitte *et al.*, 1991, Zeller and Friebe, 1991))**



## B. Origin of einkorn lineage

The einkorn lineage includes the wild species of *T. boeoticum* and various goat grasses (see Table 219). The latter were formerly considered to belong to the genus *Aegilops*, but many geneticists now classify them as belonging to the genus *Triticum*. The only domesticated species in this group is einkorn (*T. monococcum*). Species have only one grain per floret; however, they may have one or two florets per spikelet. They are diploid ( $2n = 14$ , genome AA) (Körber-Grohne 1988, Sitte *et al.*, 1991, Zeller and Friebe 1991).

Table 1.9 Geographic distribution of the diploid einkorn lineage (Körber-Grohne, 1988)

Hulled grain
Wild einkorn <i>T. boeoticum</i> (AA)
Single-grain var. <i>aegliopoides</i> (AA) Balkans, N. Greece, W. Turkey
Double-grain var. <i>thaoudar</i> (AA) E. Turkey, N. Iraq, Iran
Progeny of the two varieties (AA) Central Turkey, Transcaucasia
Goat grass <i>T. tauschii</i> ( <i>Aegilops tauschii</i> = <i>Aegilops squarrosa</i> ) (DD) Mediterranean, Central Asia, Iran, Iraq, Transcaucasia
Another five species of <i>Aegilops</i> (similar to B) Asia Minor and Central Asia
Einkorn <i>T. monococcum</i> (AA)

### C. Origin of emmer lineage

The emmer lineage includes only tetraploid hybrids with the genome AABB (see Table 1.10). The cultivated form *T. dicoccon* developed from the wild form *T. dicoccoides*. Three forms of wild emmer are found today in various parts of Asia Minor and Central Asia. Of the six domesticated species, only emmer retains its hull as a mature grain. Species have two to three florets with two grains each (Körber-Grohne, 1988; Sitte *et al.*, 1991; Zeller and Friebe, 1991).

Table 1.10 Geographic distribution of the tetraploid emmer lineage (Körber-Grohne, 1988)

Hulled grain	Naked grain
Wild emmer <i>T. dicoccoides</i> (AABB) S.E. Turkey, Israel, S. Syria, N. Iraq, W. Iran	
Wild emmer <i>T. timopheevi</i> (AAGG) Transcaucasia, Armenia, N. Iraq, W. Iran	
Wild emmer <i>T. araraticum</i> (AAGG) Transcaucasia	
Emmer <i>T. dicoccon</i> (AABB)	Durum wheat <i>T. durum</i> (AABB) N.E. Africa, Mediterranean, Spain
	Rivet/cone wheat <i>T. turgidum</i> (AABB) Portugal, UK, Spain
	Persian wheat <i>T. carthlicum</i> (AABB) Caucasia, Iraq, Iran
	Oriental wheat <i>T. turanicum</i> (AABB)
	Polish wheat <i>T. polonicum</i> (AABB) S. Europe, Turkey, Iraq, Iran, Armenia, N.W. India

### D. Origin of spelt lineage

It is assumed that genome A derives from einkorn (*T. monococcum*) and genome D from goat grass (*T. tauschii* = *Ae. squarrosa* = *Ae. tauschii*). The origin of the third genome (B) is still unclear. It possibly belongs to *Ae. speltoides* descendants or ancestors (see Section II: History of Wheat).

The hexaploid wheat group ( $2n = 42$ , genome AABBDD) is closely related to spelt, macha and the naked wheats (see Table 1.11). The genetic differences in the gene pool of hexaploid wheat are small, although they exert a considerable influence, yielding both hulled grain (*e.g.* spelt) and naked grain (wheat).

The entire hexaploid lineage (AABBDD) is regarded as a single species. The various grains (*e.g.* bread wheat *T. aestivum* ssp. *vulgare*, spelt *Triticum aestivum* ssp. *spelta*) are considered as subspecies. In practical usage, however, the earlier categories are still frequently applied (Körber-Grohne 1988).

**Table 1.11 Geographic distribution of the hexaploid spelt lineage (Körber-Grohne, 1988)**

Hulled grain	Naked grain
Macha wheat <i>T. macha</i> (AABBDD) Georgia/Transcaucasia	
<i>T. vavilovii</i> (AABBDD) Armenia	
Spelt/dinkel <i>T. spelta</i> (AABBDD)	Dwarf/club wheat <i>T. compactum</i> (AABBDD) mountains of Afghanistan, Alps
	Cake wheat ( <i>Kugelweizen</i> ) <i>T. sphaerococcum</i> (AABBDD) Afghanistan, Bukhara, N.W. India
	Bread wheat <i>T. aestivum</i> ( <i>aestivum</i> ) (AABBDD) Temperate zones

#### 4. Reproductive Biology

Reproduction of *T. aestivum* is only known in the context of cultivation (Garke 1972). Harvesting and propagation of its seed are entirely dependent on man. Wheat is predominantly self-pollinating. The cross-fertilisation rate may be as high as 1 to 2 per cent, although it can be less than 1 per cent (Poehlmann 1959). Wind-borne cross-fertilisation depends heavily on physical factors. It is minimal (0.1 per cent) where there is high humidity, but higher when there is warm, dry weather. Under such conditions, it has been claimed that the cross-fertilisation rate may be between 3.7 and 9.7 per cent. Cross-fertilisation is considerably more likely in the ears of stem branches (also called tillers) (Mandy 1970). The rate of cross-fertilisation may also depend on the variety (*e.g.* Stoner 24 to 37 per cent). Hucl (1996) shows for 10 Canadian spring wheat cultivars that the cross-pollination frequency varies according to the genotype. The frequency was always lower than 9 per cent. Apomixis is very rare (Mandy 1970).

Wheat's flowering season depends on geographical location. For example, in Germany and Sweden it flowers from late May to late June (Mandy, 1970, Garke, 1972). Flowering times for Mediterranean Europe and the centres of origin and diversity of wheat are late winter, and early spring (Galun, personal communication). Sunny weather and temperatures of at least 11 to 13°C are propitious for flowering (Mandy 1970). The inflorescence of wheat is a spike, and the ear on the main culm flowers first. The process begins in the middle third of the ear, spreading towards the tip and base. The spikelets at the top and bottom of the ear are the last to bloom (Mandy, 1970). In cultivated wheat fields, the number of ears is usually between 400 and 650/m<sup>2</sup>. Depending on the proportion of well-developed ears, the average grain count per ear varies between 35 to 40 and 20 to 25. However, the standard number of seeds per head is 30 to 35 (one ear carrying an average of 80 florets) (Kübler 1994; average data in Germany).

When flowering, the lemmas and palaeas open to an angle of 20 to 35°. The pollen sacs appear about four to six minutes later adopting a horizontal position. Under favourable weather conditions a fleret will complete the flowering cycle in 13 to 18 minutes. The reproductive organs are slightly protandrous (pollen sacs mature one to three days earlier). An unfertilised spikelet remains open for several hours or even days (Mandy 1970).

Flowering for a full ear takes between 101 and 120 hours, 23 florets a day blooming on average. Blooming begins in the early morning between 4 and 5 a.m. Peak flowering time is between 9 and 10 a.m., with a second peak between 2:30 and 3:30 p.m. By 7 p.m. flowering is usually completed. A wheat plant flowers for four to 15 days (Mandy 1970; average data in Germany).

The quantity of pollen produced by an anther is low, being approximately 2700 pollen grains per sac. It has been established that, on average, 80 per cent of pollen from an anther which protrudes from the spikelet is dispersed into the air. It was assumed from this that a wheat variety with a large number of protruding anthers would make enough pollen available to achieve cross-fertilisation. Under experimental conditions in the laboratory (moderate mass exchange of 10 g/cm per second and moderate wind speed of 3 m/sec), pollen travels about 60 m distance at a height of 1 m (D'Souza 1970). In field experiments Wilson (1968) found 10 per cent seedsetting on male sterile wheat plants that were 30 m from the pollen donor plants.

Pollen begins to germinate 15 minutes after deposition on the stigma (D'Souza 1970) and retains its fertilisation ability for only a very short period. Even under optimum conditions of 5°C and 60 per cent relative atmospheric humidity, this period will not exceed three hours. Under common field conditions of 20°C and 60 per cent relative atmospheric humidity it may remain viable for less than 30 minutes. With temperatures of about 30°C and low relative atmospheric humidity, the pollen is only able to achieve its function for 15 minutes. On hot days, therefore, this short fertilisation period can considerably reduce pollen germination in the event that cross-pollination does occur (D'Souza 1970).

## 5. Cross-fertilisation

### A. Interspecific/genus

Selection breeding, which had been ongoing for centuries, and the more recent methods of classical hybridisation breeding, have led to an enormous improvement of bread wheat traits. Biotechnological methods offer the potential to complement these traditional techniques. It has been 20 years since *in vitro* methods were first used in wheat breeding (Picard and de Buyser 1973). At that time the first variety, "Jinghua", which was produced using anther culture techniques, was licensed in China. In 1985, "Florin" became the first variety developed using *in vitro* methodology to be licensed in Europe (France) (de Buyser *et al.*, 1987, Henry and de Buyser 1990).

There are many examples of successful classical cross-breeding within the genome lineage of *T. aestivum*, and between *T. aestivum* and the other lineages described above (see Figure 1.4). Hybridisation is possible with any combination in the hexaploid lineage. The progeny are fertile because the genomes are homologous. Heterosis frequently occurs.

In general, *T. aestivum* has been used as the mother plant in inter-generic and inter-specific crossing. Many crosses have been successful, although techniques such as embryo rescue may be required to obtain viable progeny. Differences have been noted in the receptivity of different varieties of *T. aestivum* to accept cross-fertilisation by other species such as rye (Zeven 1987). One of the reasons for this is the potential control (or lack thereof) by genes Krl and Kr2 (Gale and Miller 1987).

Wheat has been the subject of considerable work involving wide crossing, but much of this will have little relevance to crosses that might occur naturally in the environment.

Crosses such as (diploid x hexaploid, tetraploid x hexaploid) reduce the fertility of the  $F_1$  generation substantially. Hybridisation is more successful if the parent with higher chromosome number is used as mother plant, although it should be noted that hybridisation between wheat x barley is efficient when barley (14 chromosomes) is used as the female parent. Most  $F_1$  hybrids from hexaploid x diploid crosses are sterile. Only manual crossing of *T. aestivum* x *T. monococcum* produced  $F_1$  hybrids with grains that germinated. Grains of the reciprocal hybrid did not germinate. When tetraploids were manually crossed with hexaploids, only the crossing of *T. aestivum* with *T. turgidum*, *T. durum*, *T. timopheevi* or *T. carthlicum* was successful (Mandy 1970, Sharma and Gill 1983). Hybrids from *T. aestivum* and *T. turgidum* are fertile. So while wheat may be crossed with many related species and some related genera,  $F_1$  plants are often highly sterile, or the embryos abort. Gene transfer occurs only through man's intervention, e.g. hand pollination, and through rescue of  $F_1$  embryos or through the use of male-sterile female plants. The chance of gene transfer occurring through such hybrids in nature is minimal. For production of genetically modified *T. aestivum*, and information about technical barriers that were overcome in achieving wheat transformation, see Appendix II.

*Triticum* species can be crossed by hand with the genera *Aegilops*, *Secale*, *Agropyron*, *Haynaldia*, *Hordeum* and *Elymus* (see Table 1.12). Trigeneric hybrids are formed in some cases (see Table 1.13). Cross-breeding with *Elymus* species has proved least successful (Poehlmann 1959, Sharma and Gill 1983, Zeller 1985, Maan 1987, Jiang *et al.*, 1994). Natural wild crosses of *T. aestivum* with the following members of the genera *Aegilops* (*Ae. cylindrica*, *Ae. triticoideis*, *Ae. neglecta*, *Ae. triuncialis*, *Ae. ventricosa*, *Ae. genicularia*, *Ae. bluncalis*, *Ae. crassa*, *Ae. juvenalis*, *Ae. speltoideis*, *Ae. tauschii* and *Ae. umbellata*) have been reported (van Slagern 1994). Crosses of *T. aestivum* to tetraploid *Aegilops* species resulted in hybrid seeds from which addition, substitution and translocation lines with introgressed genes for disease resistance have been selected (Spetsov *et al.*, 1997, Petrova and Spetsov 1997). For information about cross-breeding of wheat with *Elymus*, see Dewey (1984), Plourde *et al.*, (1989) and Koebner *et al.*, (1995); with *Thynopyrum*, see Dewey (1984) and Sharma and Baezinger (1986); with *Elytrigia*, see Dewey (1984) and Cauderon (1994); and with *Pseudoroegneria*, see Dewey (1984). Wheat can also cross with *Sorghum* and *Setaria* (Laurie *et al.*, 1990).

Most manual cross-breeding has been carried out with *Secale cereale*, in order to combine the high grain yield and protein quality of wheat with rye's disease resistance and tolerance of poor soil conditions. The resulting generic progeny is called "triticale." There are only a few reports on natural hybridisation between wheat and rye. Müntzing (1979) reports a massive natural hybridisation in 1918, resulting in up to 20 per cent male sterile  $F_1$  wheat x rye hybrids within wheat plots isolated by surrounding rows of rye plants. This spontaneous hybridisation occurred with wheat cultivars exhibiting anemophilic flower characters under dry continental conditions. In most cases, the  $F_1$  hybrids are completely male sterile and have to be pollinated by wheat, rye or fertile triticale to obtain generic progenies. Another possibility to overcome pollen sterility of wheat x rye hybrids is to double their chromosome number. Modern triticale breeding based on recombination among hexaploid triticales has solved the most important problems with the crop, namely low fertility, poor grain filling, tall stem and late ripening (Wolski *et al.*, 1996). Triticale can be exploited as a bridge for the introgression of valuable genes from *Secale cereale*, e.g. by the generation of 1B/1R translocation chromosomes. The first European cultivar of triticale was obtained in France [Clerical since 1982 and on open catalogues since 1983 (Bernard and Guedes Pinto 1980, Cauderon and Bernard 1980)].

Through the use of *in vitro* methods, dihaploid plants have been produced from crosses between wheat and *Hordeum bulbosum* (Blanco *et al.*, 1986, Cauderon and Cauderon 1956, Stich and Snape

1987) and wheat and *Zea mays* (Kisana *et al.*, 1993). In these cases, the barley and maize chromosomes are eliminated in early stages of embryo development (Barclay 1975, Laurie and Bennett 1988, 1989). After diploidisation of the resulting haploid plants, the homozygous wheat material can be used for RFLP analysis, gene localisation and isolation.

Mandy (1970) reported the first manual intergeneric hybrid between (*Triticum vulgare* x *Haynaldia villosa*) x *Secale cereale*), with the chromosome number ( $n = 35$ ). Reciprocal hybridisation has had low success.

Interspecific hybridisation under natural conditions has been reported to occur only rarely (Gotsov and Panayotov 1972).

**Table 1.12 Manual intergeneric crossing with *Aegilops* (Ae.), *Secale* (S.), *Agropyron* (A.), *Haynaldia* (Ha.), *Hordeum* (H.) and *Elymus* (E.) (Sharma and Gill, 1983)**

**Wheat parent**

Diploid wheat:

*Triticum monococcum*

**Species of allied genera crossed**

*Ae. bicornis*, *Ae. caudata*, *Ae. columnaris*, *Ae. comosa*, *Ae. cylindrica*,  
*Ae. longissima*, *Ae. mutica*, *Ae. ovata*, *Ae. speltoides*, *Ae. squarrosa*,  
*Ae. triaristata*, *Ae. tripsacoides*, *Ae. triuncialis*, *Ae. umbellulata*,  
*Ae. uniaristata*, *Ae. variabilis*, *Ae. ventricosa*

*S. cereale*

*A. elongatum*, *A. intermedium*

*Ha. villosa*

*H. vulgare*

Tetraploid wheat:

*T. turgidum*, includes

*durum*, *carthlicum*,

*dicoccum* and *dicoccoides*

*Ae. bicornis*, *Ae. biuncialis*, *Ae. caudata*, *Ae. cylindrica*,

*Ae. columnaris*,

*Ae. comosa*, *Ae. crassa*, *Ae. dichasians*, *Ae. heldreichii*, *Ae. kotschyi*,

*Ae. longissima*, *Ae. mutica*, *Ae. ovata*, *Ae. sharonensis*, *Ae. speltoides*,

*Ae. squarrosa*, *Ae. triaristata*, *Ae. tripsacoides*, *Ae. triuncialis*,

*Ae. umbellulata*, *Ae. uniaristata*, *Ae. variabilis*, *Ae. ventricosa*

*S. africanum*, *S. ancestrale*, *S. cereale*, *S. montanum*, *S. vavilovii*

*A. campestre*, *A. dasystachyum*, *A. distichum*, *A. elongatum*,

*A. intermedium*, *A. junceum* 4x, *A. obtusiusculum*, *A. repens*

*Ha. hordeace*, *Ha. villosa*

*H. brevisubulatum*, *H. chilense*, *H. vulgare*

*E. arenarius*, *E. giganteus*

Tetraploid wheat:

*T. timopheevi*

*Ae. bicornis*, *Ae. caudata*, *Ae. comosa*, *Ae. cylindrica*, *Ae. dichasians*,

*Ae. kotschyi*, *Ae. longissima*, *Ae. mutica*, *Ae. ovata*, *Ae. speltoides*,

*Ae. squarrosa*, *Ae. triuncialis*, *Ae. umbellulata*, *Ae. uniaristata*,

*Ae. ventricosa*

*S. africanum*, *S. cereale*, *S. vavilovii*

*A. campestre*, *A. cristatum*, *A. elongatum*, *A. intermedium*,

*A. junceum* 4x,

*A. repens*

*Ha. villosa*

*H. bogdanii*, *H. vulgare*, *H. vulgare* ssp. *distichon*

*Ae. bicornis*, *Ae. biuncialis*, *Ae. caudata*, *Ae. columnaris*, *Ae. comosa*,

*T. aestivum*, *Ae. crassa*, *Ae. cylindrica*, *Ae. dichasians*, *Ae. juvenalis*,

*Ae. kotschyi*,

*Ae. longissima*, *Ae. mutica*, *Ae. ovata*, *Ae. sharonensis*, *Ae. speltoides*,

*Ae. squarrosa*, *Ae. triaristata*, *Ae. tripsacoides*, *Ae. triuncialis*,

*Ae. umbellulata*, *Ae. uniaristata*, *Ae. variabilis*, *Ae. ventricosa*

*S. africanum*, *S. ancestrale*, *S. cereale*, *S. montanum*, *S. vavilovii*

*A. caespitosum*, *A. distichum*, *A. elongatum*, *A. intermedium*,

*A. junceum* 2x, *A. podperae*, *A. scirpeum*, *A. smithi*, *A. trachycaulum*,

*A. yezoense*

*Ha. villosa*

*H. chilense*, *H. pusillum*, *H. spontaneum*, *H. vulgare*, *H. vulgare* var.

*distichum*

*E. giganteus*

Hexaploid wheat:

**Table 1.13 Trigenic hybrids from manual crossing *Triticum* (T.), *Aegilops* (Ae.), *Hordeum* (H.), *Agropyron* (A.), *Haynaldia* (Ha.) and *Secale* (S.) (Sharma and Gill, 1983)**

Trigenic hybrid	Reference
( <i>T. timopheevi</i> x <i>H. bogdanii</i> ) x <i>S. cereale</i>	Kimber & Saltee 1979
( <i>H. vulgare</i> x <i>T. aestivum</i> ) x <i>S. cereale</i>	Claus 1980; Fedak & Armstrong 1980
( <i>H. vulgare</i> x <i>T. aestivum</i> ) x <i>S. montanum</i>	Claus 1980
( <i>H. vulgare</i> x <i>A. elongatum</i> ) x <i>Ae. crassa</i>	Pedigree of Sando's collection, USDA, Beltsville
( <i>T. aestivum</i> x <i>S. cereale</i> ) x <i>T. aestivum</i> x <i>A. elongatum</i>	USDA, Beltsville
Triticale (6x) x ( <i>T. durum</i> x <i>A. intermedium</i> ) amphidiploid	Nowacki <i>et al.</i> , 1979
( <i>Ae. ventricosa</i> x <i>S. cereale</i> ) x <i>T. aestivum</i>	Dosba & Jahier 1981
( <i>Ae. crassa</i> x <i>T. persicum</i> ) x <i>S. cereale</i>	Knobloch 1968
( <i>Ae. ventricosa</i> x <i>T. dicoccum</i> ) x <i>A. intermedium</i>	Knobloch 1968
( <i>Ae. ventricosa</i> x <i>T. turgidum</i> ) x <i>S. cereale</i>	Knobloch 1968
( <i>Ae. ventricosa</i> x <i>T. dicoccum</i> ) x <i>S. cereale</i>	Siddiqui 1972
( <i>T. aestivum</i> x <i>Ha. villosa</i> ) x <i>S. cereale</i>	Knobloch 1968
( <i>T. dicoccum</i> x <i>Ha. hordeacea</i> ) x <i>S. cereale</i>	Knobloch 1968
( <i>T. dicoccum</i> x <i>S. montanum</i> ) x <i>Ha. villosa</i>	Knobloch 1968
( <i>T. turgidum</i> x <i>Ha. villosa</i> ) x <i>S. cereale</i>	Knobloch 1968

## B. Introgression

Interspecific hybridisation under natural conditions has rarely occurred (Gotsov and Panayotov 1972), and the role of environmental conditions must be taken into consideration. For example, weather abnormalities may in some instances contribute to male sterility or in others to overlapping of flowering periods. Both of these factors can result in the breaking down of effective isolation barriers between species. The introgression of a new gene will also be dependent on whether or not that gene confers an ecological advantage on the recipient in specific environments. Even so, data on potential hybridisation events are helpful in assessing the potential for introgression of “novel traits” of transgenic *T. aestivum* into wild relatives. If potential “mates” of *T. aestivum* are occurring in the geographic region of interest, introgression has to be taken into consideration.

Rimpau reported observing volunteer crosses between *T. aestivum* x *S. cereale* in his wheat nursery at the beginning of this century. He called the bastard plants “mule-wheat” because they were infertile and he was not able to collect seed from them. Nevertheless, he continued to make artificial crosses (von Broock, personal communication).

Intra- and interspecific variation exists within the cytoplasm of wheat and related species, and this is important for wheat breeders. Cytoplasmic male sterility (CMS) systems are used successfully in several crops. CMS has been introduced into common wheat through interspecific and intergeneric hybridisation. Today, chloroplasts and mitochondria are subjects of molecular genetic studies and of genetic manipulation, and these techniques may in the future be used in wheat. All genetic information present in the DNA of cytoplasmic organelles is maternally inherited, and therefore the chance for gene transfer in nature is less than for nuclear genes.

## C. Interactions with other organisms

Wheat grain yield is decreased by some 50 major diseases which can produce overall crop damage (including storage damage) of 20 per cent (Spaar *et al.*, 1989). Fungal diseases are the greatest

problem. Animals, *e.g.* pigeons, crows and pheasants, feed on seeds, dig and tear out plants, or otherwise damage them. Mice, rabbits and deer can also cause considerable damage to wheat plants.

The tables in Appendix I are intended as an identification guide for categories of organisms that interact with *T. aestivum*. Clearly the organisms listed are examples, with their occurrence depending upon the geographic region where *T. aestivum* is grown.

## **6. Weed Characteristics/Weedness**

Wheat is a crop plant species with low competitive ability. It has no natural habitat outside cultivation (Garcke 1972, Tutin *et al.*, 1980). Wheat does not have high potential for weediness (Keeler 1989). Wheat plants may sometimes be found in “disturbed” areas where there is little or no competition from other “weed” species (*e.g.* waste places, fallow fields, along roadsides), but their survival at such sites is limited to short periods (Janssen *et al.*, 1995). There are no indications that wheat can become established as a self-sustaining population on a long-term basis (Sukopp and Sukopp 1993, Newman 1990).

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## APPENDIX I

Most Common Diseases and Pests in *Triticum aestivum*

Potential interactions of *T. aestivum* with other life forms during its life cycle (Wiese 1987, Spaar *et al.*, 1989, Wolff and Richter 1989, Chelkowski 1991, Cook and Veseth 1991, Wolff 1992):

**Viruses, Mycoplasmas** (See Brunt *et al.*, 1996. For more information, also see the VIDE database: <http://www.csu.edu.au/viruses/virus.html>)

Disease	Agent
Agropyron mosaic virus	Agropyron mosaic virus (AgMV), geographic occurrence <i>e.g.</i> in Eurasia, Canada and the USA
Barley stripe mosaic hordeivirus	Barley stripe mosaic hordeivirus (BSMV), geographic occurrence <i>e.g.</i> in Eurasia, Northern America, Pacific
Barley yellow dwarf virus	Barley yellow dwarf virus (BYDV), geographic occurrence world-wide; wheat varieties show different tolerance level (Baltenberger <i>et al.</i> , 1987); tolerance level had been increased through cross breeding with resistant <i>Agropyron</i> varieties (Ohm <i>et al.</i> , 1989, Gondart <i>et al.</i> , 1993)
Barley yellow streak mosaic virus	Barley yellow streak mosaic virus, geographic occurrence <i>e.g.</i> in Canada and USA
Barley yellow striate mosaic cytorhabdovirus	Barley yellow striate mosaic cytorhabdovirus (BYSMV), geographic occurrence <i>e.g.</i> in Africa, Eurasia, Middle East and Pacific
Brome mosaic virus	Brome mosaic virus (BMV), geographic occurrence <i>e.g.</i> in Eurasia, Australia, South Africa and USA
European striped wheat mosaic	Probably mycoplasmas
Wheat American striate mosaic nucleorhabdovirus	Wheat American striate mosaic nucleorhabdovirus (WASMV), geographic occurrence <i>e.g.</i> in Canada and USA
Wheat dwarf virus	Wheat dwarf virus (WDV), geographic occurrence <i>e.g.</i> in Bulgaria, former Czechoslovakia, Hungary, former USSR, France and Sweden
Wheat European striate mosaic tenuivirus	Wheat European striate mosaic tenuivirus (EWSMV), geographic occurrence <i>e.g.</i> in Czech Republic, Poland, Romania,

	Denmark, Finland, Sweden, Germany, UK and Spain
Wheat soilborne mosaic virus	Wheat soilborne mosaic virus, geographic occurrence <i>e.g.</i> in China, Japan, Italy and USA
Wheat spindle streak mosaic virus	Wheat spindle streak mosaic virus, (WSSMV), geographic occurrence <i>e.g.</i> in France, Germany, Italy, India, Japan, China, and USA
Wheat spindle streak virus	Wheat spindle streak virus
Wheat streak mosaic virus	Wheat streak mosaic virus (WSMV), geographic occurrence <i>e.g.</i> in Canada, USA, Romania and Jordan
Wheat striate mosaic virus	Wheat striate mosaic virus
Wheat yellow leaf virus	Wheat yellow leaf virus (WYLV), geographic occurrence <i>e.g.</i> in Japan and Italy
Wheat yellow mosaic brymovirus	Wheat yellow mosaic brymovirus, geographic occurrence <i>e.g.</i> in China, Japan, Korea, Canada and France
Wheat yellow mosaic virus	

**Bacteria**

Disease	Agent
Basal glume blotch	<i>Pseudomonas syringae</i> pv. <i>atrofaciens</i> (McCulloch)
Black glume	<i>Xanthomonas campestris</i> pv. <i>translucens</i> (Jones, Johnson et Reddy) dye Various known forms which differ only in host specificity: <i>undulosa</i> , <i>cerealis</i> , <i>hordei</i> , <i>secalis</i> , <i>oryzicola</i> and <i>phleipratensis</i>

**Fungi**

Disease	Agent
Ergot	<i>Claviceps purpurea</i> : infects florets and produces grain-like sclerotia containing mycotoxins (ergot alkaloids). The fungal grains are harvested with the wheat grains and, if not removed, mycotoxin contamination of products occurs.
Eyespot, stembreak, straw breaker	<i>Pseudocerospora herpatrichoides</i> (Fron.) Deight., Syn.: <i>Cerospora herpotrichoides</i> (Fron.), breeding for resistance; wheat genotypes with short shoot and good steadiness

<i>Fusarium</i> diseases of shoots (root and culm rots, partial head blight)	<p>Numerous <i>Fusarium</i> species play a part in the pathology of the cereal fusaria. The major species are:</p> <ul style="list-style-type: none"> <li>– <i>Fusarium nivale</i> (Ces., Syn.: <i>Geltrachia nivalis</i>)</li> <li>– <i>Fusarium culmorum</i> (W.G. Smith) Sacc. var. <i>culmorum</i></li> <li>– <i>Fusarium avenaceum</i> (Fr.) Sacc. var. <i>avenaceum</i></li> <li>– <i>Fusarium graminearum</i> Schwabe (perfect form: <i>Gibberella zeae</i> (Schw.) Petch): widespread, especially harmful not only to wheat but also to maize</li> <li>– <i>Fusarium poae</i> (Peck) Wollenw.: occurs sporadically, often in conjunction with the grass mite (<i>Siteroptes graminum</i> [Reuter]), which feeds on the fungus and helps it to proliferate.</li> <li>– Other species found in wheat include: <i>Fusarium acuminatum</i> Ell. et Kellerm. (<i>Gibberella acuminata</i> Wollenw.), <i>Fusarium dimerum</i> Penzig, <i>Fusarium equiseti</i> (Corda) Sacc. (<i>Gibberella intricans</i> Wollenw.), <i>Fusarium porotrichoides</i> Sherb., <i>Fusarium tricinctum</i> (Corda) Sacc. and <i>Fusarium moniliforme</i> Sheldon sensu Wollenw. et Reinking, increased resistance breeding in wheat; chemical treatment led to unsatisfactory results (Maurin <i>et al.</i>, 1996).</li> </ul>
Glume blotch (Septoria disease)	<p><i>Leptosphaeria nodorum</i> (E. Müll.), conidial form <i>Septoria nodorum</i> Berk., Syn.: <i>Phaeosphaeria nodorum</i> (E. Müll.) Hejarude, only partial resistance in wheat found (Jeger <i>et al.</i>, 1983, Bostwick <i>et al.</i>, 1993).</p>
<i>Helminthosporium</i> yellow blotch disease	<p><i>Drechslera tritici-repentis</i> (Died.) Shoem., perfect form: <i>Pyrenophora trichostoma</i> (Fr.) Fckl., Syn.: <i>Pyrenophora tritici-repentis</i> (Died.) Drechsler.</p>
Mould	<p><i>Aspergillus</i> ssp./<i>Penicillium</i> ssp. can proliferate during storage. Both are potential mycotoxin producers (Ochratoxin A).</p>
<i>Phoma</i> leaf spot	<p><i>Phoma glomerata</i> (Cda.) Wr. et Hochaf.</p>
Pointed eyespot (stem break, straw breaker)	<p><i>Rhizoctonia</i> spp., <i>Thanatephorus cucumeris</i> (Frank) Donk.</p>

Powdery mildew of cereals	<i>Erysiphe graminis</i> DC. f. sp. <i>tritici</i> March, resistance genes, e.g. Mlk, Pm1 to Pm9, M1Ax, U1 and U2, can be found in different wheat varieties and related species (Heun and Fischbeck 1987, 1989, Hovmoller 1989, Zeller <i>et al.</i> , 1993).
Rusts	
Yellow/stripe rust	<i>Puccinia striiformis</i> (West., Syn.: <i>Puccinia glumarum</i> Erikss. et Henn). Formation of pathotypes which specialise in wheat or barley. In exceptional cases wheat stem rust strains may attack highly susceptible barley varieties or vice versa.
Leaf rust of wheat	<i>Puccinia recondita</i> Rob. ex Desm. f. sp. <i>tritici</i> , Syn.: <i>Puccinia triticina</i> Erikss., Syn.: <i>Puccinia rubigovera</i> Wint. Formation of pathotypes, alternate host <i>Thalictrum</i> spp.
Black stem rust of wheat	<i>Puccinia graminis</i> Pers. f. sp. <i>tritici</i> Development of formae speciales specialised in rye, barley, oats, wheat and grasses. Numerous pathotypes formed.
Septoria leaf blotch	<i>Mycosphaerella graminicola</i> (Fckl.) Sanderson, conidial form: <i>Septoria tritici</i> Rob. ex Desm.
Smuts	
Loose smut of wheat	<i>Ustilago tritici</i> (Pers.) Rostr.
Covered smut of wheat	Various <i>Tilletia</i> species with different sori, including: – <i>Tilletia caries</i> (DC.) Tul. Syn.: <i>Tilletia tritici</i> (Bjerk.) Wint. – <i>Tilletia foetida</i> (Wallr.) Liro, Syn.: <i>Tilletia laevis</i> Kühn or <i>Tilletia foetens</i> (Bjerk. et Curt.) Schroet. – <i>Tilletia intermedia</i> (Gassner) Savul. Syn.: <i>Tilletia tritici</i> f. sp. <i>intermedia</i> Gassner <i>Tilletia controversa</i> Kühn <i>Neovossia indica</i> (Mit.) Mund. <i>Urocystis agropyri</i> (Preuss.) Schroet.
Dwarf bunt of wheat	<i>Gaeumannomyces graminis</i> (Sacc.) v. Arx. et Olivier var. <i>tritici</i> Walker
Carnal smut	Several varieties with overlapping hosts, var. <i>tritici</i> attacks wheat, triticale, barley and rye, no resistant varieties in wheat found.
Stripe/flag smut	
Take-all	

**Animals**

<b>Pest</b>	<b>Agent</b>
<p>Apart from the above-mentioned species of aphid, the following species may cause damage to cereals, maize and grasses:</p>	<p>Bromegrass aphid (<i>Diuraphis bromicola</i> [H.R.L.]), cat's-tail aphid (<i>Diuraphis mählei</i> [Börn.]), corn leaf aphid (<i>Rhopalosiphum maidis</i> [Fitch.]), yellow cherry/reed canary grass aphid (<i>Rhopalomyzus lonicerae</i> [Siebold]), <i>Rhopalomyzus poae</i> [Gill.], cocksfoot aphid (<i>Hyalopteroides humilis</i> [Walk.]), <i>Laingia psammae</i> (Theob.), <i>Schizaphis nigerrima</i> H.R.L., <i>Metopolophium festucae</i> (Theob.), green grain aphid (<i>Schizaphis graminum</i> [Rond.]), grain aphid (<i>Sitobion-granarium</i> [Kirby]), eob-aphid (<i>Sipha maydis</i> [Pass.]), <i>Sipha glyeriae</i> [Kalt.]), black (bean) aphid (<i>Aphis fabae</i> Scop.), green peach aphid <i>Myzus persicae</i> [Sulz.])</p>
<p>Aphids:</p> <p>Grain aphids</p> <p>Oat or bird cherry aphid</p> <p>Rose grain aphid</p>	<p>Aphids arrive from early May (when wheat is shooting), settling first on leaf blades and sheaths, transferring to inflorescence as ears extend. Warm and dry conditions encourage generations. The generation cycle lasts 8 to 10 days. Each aphid can lay 30 to 50 larva (parthenogenesis). Around mid-July mass proliferation is briefly interrupted due to poor feeding conditions and the appearance of parasites and predators (ladybirds/ladybugs). The grain aphid undergoes a holocycle, i.e. sexual differentiation takes place in autumn, and winter eggs are laid on grasses. More than 10 generations occur in the space of a year. <i>Macrosiphum avenae</i> (Fabr.), Syn.: <i>Sitobion avenae</i> (Fabr.)</p> <p>Also in barley, oats, rye, maize, fodder grasses</p> <p>Aphid species which does not alternate hosts <i>Rhopalosiphum padi</i> (L.)</p> <p>Alternate-host aphid with broad host plant profile among cereal and grass species, e.g. barley, oats, maize, fodder grasses.</p> <p><i>Metopolophium dirhodum</i> (Walk.)</p> <p>Alternate-host aphid (also in barley, oats, rye, maize, fodder grasses).</p>

<p>Cereal cyst nematodes, cereal stem eelworm</p>	<p><i>Heterodera avenae</i> Woll. Also attacks barley, oats, rye, fodder grasses. Several biotypes distinguished by their host profile. Cysts drop from roots and survive in soil. Larvae hatch in spring and infect roots. Sexual differentiation occurs in the root. Females carry up to 600 eggs. When a female dies, its body turns brown and is transformed into a lemon-shaped cyst, only limited resistance (Cre 1 gene on chromosome No. 2B) found in wheat (Slootmaker <i>et al.</i>, 1974).</p>
<p>Cereal leaf beetle</p>	<p>Red-throated cereal leaf beetle (<i>Oulema melanopus</i> [L.], Syn.: <i>Lema melanopa</i> [L.]), blue cereal leaf beetle (<i>Oulema lichenis</i> [Voet], Syn.: <i>Lema lichenis</i> [Voet]) Beetles leave winter quarters in mid-April and migrate into cereal fields. Eggs are laid in late May on upper side of leaves. This takes 6 to 8 weeks. Each female lays 50 to 100 eggs. Egg development lasts 7 to 14 days.</p>
<p>Corn beetle</p>	<p><i>Zabrus tenebroides</i> Goeze (corn ground beetle) Beetles appear in late June to early July. Eggs are laid in August and September. Each female lays 80 to 100 eggs in the soil. The first larvae hatch after 14 days and undergo three stages. Overwintering is in the 1st or 2nd larval stage. At soil temperatures of -1°C in spring they resume feeding. The bulk of damage now occurs. Soil pupation takes place in May. The generation cycle of the corn ground beetle lasts one year. Also found in barley, oats, rye, maize, fodder grasses.</p>
<p>Crane-fly larvae</p>	<p>Larvae of the marsh crane-fly (<i>Pales (Tipula) paludosa</i> Meig.), common crane-fly (<i>Pales (Tipula) oleracea</i> L.), autumn crane-fly (<i>Pales (Tipula) czizeki</i> de Jong). Biggest factor: <i>Pales paludosa</i>. Also in barley, oats, rye, maize, fodder grasses.</p>

March fly larvae	<i>Bibio hortulans</i> (L.), <i>Bibio marci</i> (L.), <i>Bibio johannis</i> (L.), <i>Bibio clavipes</i> (Meig.) Also in barley, oats, rye, maize, fodder grasses.
Myriapods	Various species of myriapods, notably the common millipedes <i>Cylindroiulus</i> <i>teutonicus</i> (Pocock) and <i>Blaniulus</i> <i>guttulatus</i> (Bosc.) Also in barley, oats, rye, maize, fodder grasses.
Root aphids	<i>Anoecia corni</i> (Fabr.), <i>Anoecia vagans</i> (Koch), <i>Aploneura graminis</i> (Buckt.), <i>Aploneura lentisci</i> Pass., <i>Byrsocrypta</i> <i>personata</i> Börner, <i>Forda marginata</i> Koch, <i>Forda formicaria</i> V. Heyden, <i>Geoica discreta</i> Börner, <i>Tetraneura</i> <i>ulmi</i> (L.) Also in barley, oats, rye, maize, fodder grasses
Slugs	Various species of slug, notably the field slug ( <i>Deroceras reticulatum</i> O.F. Müll., <i>Deroceras agreste</i> L.), the garden/blackfield slug ( <i>Arion hortensis</i> [Fér.], <i>Arion rufus</i> [L.]). Also in barley, oats, rye, maize, fodder grasses.
Wheat and grass bugs	Wheat and grass bugs are a non-homogeneous group of pests. The greatest economic damage is caused by wheat bugs ( <i>Eurygaster</i> spp.). Also in barley, oats, rye, maize, fodder grasses.
Wheat nematodes	<i>Anguina tritici</i> (Steinbuch) Filipjev The larvae which live in the galls can be preserved for years in dried state.

NOTE: A complete list of US wheat pests can be found on the American Phytopathology Society home page:  
<http://www.scisoc.org/resource/common>

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## APPENDIX II

### Transformation of *Triticum aestivum*

The genetic improvement of cereals, including wheat, has been a major focus of plant breeding efforts during the past 50 years. It has resulted in remarkable increases in yield as well as improvements in quality. Nonetheless, plant breeding is a slow process and has biological limitations. In this context the rapidly emerging technologies of plant cell and molecular biology, by permitting access to a much wider gene pool, have attracted much attention, for they provide powerful and novel tools to supplement and complement the traditional methods of plant breeding.

Modern plant biotechnology is based on the delivery, integration and expression of defined foreign genes into plant cells which can then be grown *in vitro* to regenerate plants. The efficient regeneration of normal fertile plants from protoplasts is a basic prerequisite for this technology. For gramineous species, the *in vitro* regeneration of fertile phenotypically normal plants has been very difficult (Vasil and Vasil 1992). The greatest problem to overcome was that of culturing immature and undifferentiated tissue and organ explants at defined development stages in special nutrient media. Now all important cereals, *e.g.* wheat, barley, rice, can be regenerated from cultured tissue as well as single cells (Vasil 1994). Most early attempts to transform cereals were limited to the use of totipotent embryogenic protoplasts, but embryogenic protoplast cultures are difficult to establish and maintain. For wheat, *in vitro* regeneration from immature embryos from young inflorescences and microspores (somatic and gametic embryogenesis) has been possible for some time. However, to provide the cells with the greatest access to the transgenes, and in order to obtain cell culture homogeneity, it seems necessary to achieve genetic transformation of cereals using isolated single cells. In this way, it has been thought that the occurrence of chimaeric transformants would also be avoided. This strategy has been successful with many plant species (both dicots and monocots such as rice and maize). Today, normal and fertile plants can be regenerated from all major species of cereals, including wheat (Vasil *et al.*, 1990). However, it is still an inefficient, time-consuming procedure (Vasil and Vasil 1992).

There are different methods of delivering foreign genes into plants (see review: Nehra *et al.*, 1995). The well known, and often preferred method of *Agrobacterium*-mediated transformation does not work very well with cereals. Like most monocotyledonous species, wheat is generally considered to be outside the natural host range of the *Agrobacterium* pathogen. Experiments with wheat and maize have shown that *Agrobacterium* can transfer viral genomic sequences to cereal cells, resulting in a systemic viral infection called "agroinfection" (Smith and Hood 1995). For this to occur, it is not necessary to achieve integration of the viral genes into the plant genome. Thus it seems that the main difficulty is not the delivery of DNA, but rather its integration (Grimsley *et al.*, 1987, Dale *et al.*, 1989). Recent data from experiments with rice (Hiei *et al.*, 1994), maize (Ishida *et al.*, 1996), barley (Tingay *et al.*, 1997) and also wheat (Chen *et al.*, 1996) showed efficient transformation mediated by *Agrobacterium*, with stable integration, expression and inheritance of the transgenes (Chen *et al.*, 1997).

Two methods, involving osmotic (polyethylene glycol treatment) or electric (electroporation) shock, have been used for transformation and have resulted in transient as well as stable expression of the introduced gene (review: Lörz *et al.*, 1985), *e.g.* of maize (Fromm *et al.*, 1986). For wheat transformation the biolistic method was used (Vasil *et al.*, 1992, Weeks *et al.*, 1993, Becker *et al.*,

1994, Nehra *et al.*, 1994). This procedure is based on the high-velocity bombardment of plant cells with DNA-coated microprojectiles, accelerated by gunpowder discharge or pressurised helium gas (Sanford *et al.*, 1991, Klein *et al.*, 1992). The main advantage of this method is its ability to deliver DNA into intact regenerable (via the formation of somatic embryos) plant cells, eliminating the need for protoplasts, which thus minimises the potential for tissue culture effects and the resulting abnormalities (Vasil *et al.*, 1993, Vasil 1994).

Optimum expression of genes in the target cell is important for achieving a high frequency of stable transformation. In wheat, considerable efforts have been made in developing suitable gene expression vectors for transformation (Nehra *et al.*, 1995). The inclusion of an intron between the promoter and the coding region proved useful to achieve enhanced transient gene expression in wheat (Chibbar *et al.*, 1991). Furthermore, the isolation of monocot gene promoters, such as the rice actin (Act1) promoter (McElroy *et al.*, 1991) or the maize ubiquitin (Ubi1) promoter (Christensen *et al.*, 1992) sometimes resulted in higher expression frequency. Transgenic wheat has been produced using both promoters (Weeks *et al.*, 1993, Nehra *et al.*, 1994).

To obtain transgenic plants from the few stably transformed cells achieved through these transformation techniques, a suitable selection system is required. Selectable marker genes that confer resistance to antibiotics or herbicides are usually used. Among the various antibiotic resistance marker genes in use, the kanamycin resistance gene has proven ineffective for selection of transformed wheat cells because these cells and the wheat tissue itself both have a high level of endogenous tolerance to kanamycin. Another problem is that using this antibiotic as the selection agent interferes with plant regeneration (Hauptmann *et al.*, 1988, Peng *et al.*, 1992). Geneticin (G 418), however, another member of the aminoglycosides, can be effectively used (Nehra *et al.*, 1994). Hygromycin was used by Hauptmann *et al.*, (1988) with a positive result, but experiments conducted by Nehra *et al.*, (1995) were not successful. As an alternative to antibiotic resistance marker genes, genes conferring resistance to herbicides such as glufosinate ammonium (l-phosphinothricin) can be used (Nehra *et al.*, 1995). Detailed descriptions of the available monocot selection marker systems were presented in the following reviews: Wilmink and Dons 1993, McElroy and Bretzell 1994.

In recent years there have been releases of transgenic wheat plants (see Table II-1). For more information about this topic in Europe, see RKI, the SNIF database (<http://www.rki.de>) and the list of "SNIF circulated under article 9 of Directive 90/220/EEC XI/559/94-Rev 6". For the United States, the reviews of James and Krattinger 1996 and de Kathen 1996, and the APHIS ISB environmental release database (<http://www.aphis.usda.gov/bbep/bp>) provide similar information. The OECD BioTrack database includes information on experimental releases to the environment of genetically modified plants and micro-organisms (<http://www.olis.oecd.org/biotrack.nsf>).

Future advances in the molecular improvement of wheat, as in that of other plants, will depend upon the limited availability of agronomically important genes more than on any other factor. Attention is being directed to the development of DNA-based maps of wheat for identifying, and then characterising and cloning, genes of importance and interest. Gill *et al.*, (1991), for example, provided a standard karyotype and nomenclature system for describing chromosome bands in bread wheat, while Hohmann *et al.*, (1994) prepared a genetic/physical map of group 7 chromosomes. Devos and Gale (1992) tested the use of random amplified polymorphic DNA (RAPD) markers. They were unsuccessful because of the non-homologous, non-dose responsive and dominant behaviour of RAPD products. Vaccino and Metakovsky (1995) used RFLP patterns of wheat gliadin alleles as markers, and Devos *et al.*, (1995) used microsatellite sequences. Genetic maps, gene markers and QTL are now becoming available or are being developed. This work started in 1985 at the Plant Breeding Institute and the John Innes Centre in the UK, at universities in the United States, and at the INRA in France (Nelson *et al.*, 1995a, 1995b, Cadalen *et al.*, 1996).

Molecular improvement of wheat for multigenic traits, such as yield, will be a difficult and lengthy process (Vasil 1994). However, the conservation of gene order along chromosomes, as well as the similarity of gene composition and map collinearity in cereals, should be a great advantage in regard to the identification and cloning of important genes (Bennetzen and Freeling 1993, Kurata *et al.*, 1994).

#### Deliberate releases of transgenic wheat

Country	First release	Main trait
UK	1994	marker
UK	1994	herbicide resistance (glufosinate)
UK	1995	herbicide resistance (glufosinate)
UK	1995	improved starch quality
UK	1996	pest resistance (tolerance to leaf fungal disease)
Spain	1996	herbicide resistance (glufosinate), improved starch quality
UK	1997	alteration in baking quality
Belgium	1997	male sterility/restorer
Argentina	1993	improved quality, male sterility, marker
Argentina	1995	herbicide resistance
Chile	1995	herbicide resistance
USA	1994	herbicide resistance
USA	1994	herbicide resistance (glufosinate)
USA	1994	herbicide resistance (glyphosate)
USA	1995	fungal resistance
USA	1995	herbicide resistance
USA	1995	virus resistance
USA	1995	improved quality
USA	1996	fungal resistance
USA	1996	improved quality
USA	1996	fungal resistance
USA	1996	fungal resistance (glyphosate)
USA	1996	improved quality
USA	1996	herbicide resistance
USA	1996	virus resistance (glyphosate)
USA	1996	herbicide resistance
USA	1996	fungal resistance (glyphosate)
USA	1996	fungal resistance

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## SECTION 8

### SUGAR BEET (*BETA VULGARIS* L.)

#### 1. General Description Including Taxonomy, Morphology, Genetic Characteristics and Use as a Crop Plant

##### A. Taxonomy

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima*) belongs to the family *Chenopodiaceae* and the genus *B. vulgaris* comprises several cultivated forms of *B. vulgaris* subsp. *vulgaris*. Cultivars include leaf beet (var. *cicla*) and beetroot (root beet USA). The genus *Beta* is divided into four sections shown in Table 1.14 below (Ford-Lloyd and Williams, 1975; Campbell, 1976; Tranzschel, 1927 and Ulbrich, 1934):

**Table 1.14 Classification of the *Beta* species**

Species name	Chromosome number
<b>Section I: <i>Beta</i> Tranzschel</b>	
<i>B. vulgaris</i> L. ssp. <i>vulgaris</i>	18
<i>B. vulgaris</i> L. ssp. <i>maritima</i> (L.) Arcang	18
<i>B. vulgaris</i> L. ssp. <i>adanensis</i> (Pam.) Ford-Llyod & Williams	18
<i>B. patula</i> Ait.	18
<i>B. macrocarpa</i> Guss.	18, 36
<b>Section II: <i>Corollinae</i> Ulbrich</b>	
<i>B. macrorrhiza</i> Stev.	18
<i>B. corolliflora</i> Zoss.	36
<i>B. lomatogona</i> F. et M.	18, 36
<i>B. intermedia</i> Bunge	36, 45
<i>B. trigyna</i> W. et K.	36, 54
<b>Section III: <i>Nanae</i> Ulbrich</b>	
<i>B. nana</i> Boiss. et Heldr.	18
<b>Section IV: <i>Procumbentes</i> Ulbrich</b>	
<i>B. procumbens</i> Chr. Sm.	18
<i>B. webbiana</i> Moq.	18
<i>B. patellaris</i> Moq.	18, 36

In Europe, wild sea beet occurs as a wild plant. Wild *B. vulgaris* species are distributed along the border-zones of the Mediterranean from southern Russia, the Near-East, and Syria to the Canary Islands and Madeira. They are also found along the European Atlantic coasts where they come into contact with the Gulf Stream. *B. vulgaris* has also been introduced into Baltic and Central and South

America. In North America, the species has become naturalised, resulting from the introduction of plants for cultivation. Table 1.16 shows the global distribution of the wild species of *Beta*.

**Table 1.15 Distribution and use of cultivated forms of *Beta vulgaris* ssp. *Vulgaris* (according to Mansfeld, 1986)**

Species	Var.	Common name	Distribution	Use
<i>Beta vulgaris</i>	<i>Cicla</i>	Spinach beet	Central, western and southern Europe Asia	Cooking vegetable
<i>Beta vulgaris</i>	<i>Flavescens</i>	Swiss chard	Central, western and southern Europe Asia	Cooking vegetable
<i>Beta vulgaris</i>	<i>Vulgaris</i>	Red beet (beetroot)	Central, western and southern Europe; Asia; Western India	Cooking and salad vegetable
<i>Beta vulgaris</i>	<i>Lutea</i>	Yellow beet	Central, western and southern Europe; Asia	Salad vegetable
<i>Beta vulgaris</i>	<i>Rapacea</i>	Fodder beet	Europe; Commonwealth of independent States (CIS); North America	Fodder plant
<i>Beta vulgaris</i>	<i>Altissima</i>	Sugar beet	Europe; CIS; China; Asia; North America; South America	Beet sugar production

**Table 1.16 Global distribution of the wild species of the genus *Beta* (according to Mansfeld, 1986)**

Species	Subspecies	Distribution
<i>Beta vulgaris</i>	<i>maritima</i>	Mediterranean, Canary Islands, Near East, Madeira, European Atlantic coast to India, North sea, Middle and South America
<i>Beta vulgaris</i>	<i>adanensis</i>	Mediterranean, Canary Islands, Near East, Madeira, European Atlantic coast to India, North sea, Middle and South America
<i>Beta macrocarpa</i>		India
<i>Beta patula</i>		Mediterranean, western Europe, North-west Africa
<i>Beta vulgaris</i>	<i>adanensi</i>	Mediterranean, western Europe, North-west Africa
<i>Beta intermedia</i>		Asia Minor to Hungary, Persia
<i>Beta corolliflora</i>		Asia Minor, Caucasus, Black sea coast
<i>Beta macrorhiza</i>		Asia Minor, Caucasus, Black sea coast
<i>Beta trygina</i>		Asia Minor, Caucasus, Black sea coast
<i>Beta patellaris</i>		North-west African coast and Islands, southern Spain
<i>Beta procumbens</i>		Canary and Cape Verde Islands, North-west African coast
<i>Beta webbiana</i>		Canary and Cape Verde Islands, North-west African coast

Sugar beet is cultivated world-wide, but primarily in warm and temperate climates with little precipitation. There is an increase in cultivation in subtropical regions (Brouwer *et al.*, 1976). The largest areas of cultivated sugar beet are in the U.S.A., C.I.S. (Commonwealth of Independent States, formerly the U.S.S.R. [e.g. Russia]), Europe (FAO Yearbook, 1992) and in China.

## B. Uses

Sugar beet is used for the production of sugar. By products of sugar production as pulp, molasses, fibre etc, are used as feed.

When sugar beet is grown in areas of livestock production, leaves of the plant may also be used for fodder. More recently, sugar beet has been used for molasses production. Molasses are used for alcohol production and in other forms of fermentation (penicillin production, etc...).

## C. Description

### *Morphology*

A glabrous or slightly, hairy annual, biennial or perennial of very varied habit, from 30 to 120 cm (or even 200 cm) in height. The root is stout, sometimes conspicuously swollen forming a beet together with the hypocotyl, and sometimes forming a branched taproot (as in *ssp. maritima*). Stems are decumbent, ascending or erect, and more or less branched. Leaves are very varied in size, shape and colour, often dark green or reddish and rather shiny, frequently forming a radicle rosette. Inflorescences are usually large and more or less branched. The flowers are hermaphrodite arranged in small cymes (Clapham *et al.*, 1962; Højland and Pedersen, 1994).

Cultivated forms of sugar beet are essentially biennial and are grown for the swollen roots that develop at the end of the first growing season. Sugar beets is biennial and require a period of vernalisation at the end of the first year before they can flower, although a small proportion of plants flower in their first year and are able to set seeds that persist in the soil. This phenomenon is known as "bolting". A possible source of annual weed beets is the pollination of seed crops by contaminating pollen from annual wild beets (Longden, 1976; Evans and Weir, 1981). In particular, this may have happened in southern Europe during the production of sugar beet seed of triploid monogerm varieties, when the male-sterile diploids used as mother plants are especially susceptible to pollination by contaminating pollen from diploid wild or weed beet plants, rather than by the intended tetraploids pollen bearing plants (Scott R.K. and Longden P.C., 1970). The other possibility is the variability in vernalisation requirements between varieties, some varieties need less vernalisation than the other and can easily flower during the first year.

In Europe, flowering weed beets in sugar beet production areas have, since the early 1970's, become a serious problem. The weed beet is phenotypically different from volunteer sugar beet in that it produces more seed, and in France, this seed has been shown not to require the usual vernalisation period prior to flowering (Harding and Harris, 1994). The weedy form may, in theory, have evolved in parallel with "bolters" *in situ* in sugar beet producing areas, but, molecular evidence suggests that weed beet originated from pollination by wild diploid species in seed producing areas along the Mediterranean (Boudry *et al.*, 1992; 1993).

*Beta vulgaris ssp. vulgaris* is customarily divided into two types: fodder beet and sugar beet. Some authors refer to sugar beet as var. *saccharifera*, however the distinction is not clear. The obvious morphological difference is that the beet in fodder beet is formed primarily by the hypocotyl, whereas in the sugar beet a considerable part of the beet is formed by the root. This results in a higher dry matter content in sugar beet, and also the beet itself is placed deeper in the soil. A variety of beet is, as a rule, only accepted as a sugar beet if the dry content matter is 20% or more and the beet is white (Højland and Pedersen, 1994).

The wild *Beta vulgaris* ssp. *maritima* is hardy, has thin, multi-stemmed roots, and low-lying stalks in a rosette-like array.

#### *Seedlings*

Sugar beet seeds contain very little perisperm for germination and early growth. This makes seedlings very vulnerable during early growth to competition from weeds and to damage by disease and browsers (Højland and Pedersen, 1994). Weeds emerging within 4 weeks after the sugar beet has reached the two-leaf stage are the most damaging. Weed competition has been estimated to reduce root yields 6 % in Canada and 10% in the USA. Competition from annual grasses also suppress root yields, however, competition from annual grass species is not usually as severe as that from broadleaf weeds because they do not compete for light as effectively as broadleaf weeds (Højland and Pedersen, 1994). Sugar beet seedlings have two, and occasionally three leaves, however, varieties vary in terms of leaf position, leaf number, leaf size, and curling of the leaf edge. Yellow and red pigments are often stored in leaf tissue. After differentiation of the leaves, they become covered with a waxy layer (Gilloly Bystron *et al.*, 1968). Unlike fodder beet, the lower leaves of the sugar beet commonly lie in a rosette-form on the ground (Brouwer *et al.*, 1976). The crown leaves are spirally arranged in 5/13 position. Leaf development is usually most advanced from the end of July until late August, depending on the area of cultivation. Leaf yellowing and wilting generally begins when temperatures drop below 6°C.

#### *Plant development*

*Beta vulgaris* is hemocryptophytic, that is it is a plant that develops its buds just above, or below the soil-surface where they are protected from drought or cold experienced during very cold winters (Højland and Pedersen, 1994).

Sugar beets generally only show stem elongation in the second growing season although, as previously noted, this may begin in the first year in some plants (bolting). The gene B located on chromosome 2 (Boudry *et al.*, 1994) cause shoot elongation and early flowering without vernalisation. The dominant allele fragmentally occur in *B. maritima* populations. Also quantitative genetic variation for bolting has been observed resulting in stem elongation under specific environmental conditions. The most important of these are low temperatures (+1 to +4°C) in the 4-5 leaf stage, the duration of low temperature, day length, and the effects on the phytochrome system of light quality (Lane *et al.*, 1965; Lexander, 1981; Smit, 1983). Due to successes in breeding programmes, today's cultivated sugar beet varieties show very little stem elongation ("bolting") in the first year.

#### *Root*

The fibrous root system can reach to a depth of 1-2- meters. Numerous secondary roots spread out directly under the soil surface and are highly branched. Secondary roots submerged deeper in the soil are stronger and grow in downward arcs. 70% of the root mass is located in the soil layer from 0-30 cm. The roots of cultivated beets range in colour from white to yellow, orange and red in various shades and intensities. Sugar beets are sometimes "fangy"; this refers to overdeveloped secondary roots alongside the taproot.

### **D. Genetic characteristics (ploidy number)**

The genus *Beta* exists in diploid, tetraploid and hexaploid forms with a chromosome number of  $x=9$  (Walter, 1963) (see also Table 1.14). Dense genetic maps based on molecular marker have been published and linkage groups have been allocated to the 9 chromosomes of beet (Barzen *et al.*, 1992;

Pillen *et al.*, 1992). also the abundance of repetitive sequence classes has been extensively studied (Schmidt and Heslop-Harrison, 1993). All wild and cultivated *Beta* species are capable of hybridising, and wild beet species represent a valuable gene reservoir and are frequently used in variety breeding programmes.

Most of the sugar beet grown since 1970s has been triploid hybrids, although actually the diploid varieties represent 50 % in France. Triploid plants are produced by crossing a tetraploid male parent, onto a diploid male sterile plant, used as the female parent. The resulting plants are usually doubly sterile because of chromosome imbalance and cytoplasmically inherited male sterility in the same plant. However, small proportions of plants do produce aneuploid pollen, which will give fertile progeny when used to pollinate the diploid male sterile plants.

The development of hybrid sugar beet was made possible by the discovery of cytoplasmic male-sterility (CMS) (OECD, 1993b). As in other plant species, CMS in sugar beet is the result of the interaction between nuclear genes and changes in the mitochondrial genome. To obtain entirely male sterile offspring, CMS plants must be pollinated with so-called maintainer plants, which carry the normal, unchanged mitochondrial genome (OECD, 1993b).

Truly nuclear male-sterility which depends on a single recessive nuclear gene exist but this system does not allow the production of a population that is 100% male-sterile (OECD, 1993b).

The goal of breeding programmes is to develop sugar beet varieties with higher root yield and higher sugar content, better extraction yield (juice purity), higher seed germination percentages; lower tendency to "bolt"; physical attributes of the root well adapted to mechanical harvesting; higher resistance to leaf diseases; and, higher root dry matter content (especially for fodder beet).

#### **E. Survival strategies**

Sugar beet possesses long-lived dormant seeds that can become a volunteer weeds in sugar beet fields (Højland and Pedersen, 1994). They tend to germinate in the field 1-3 days later than planted sugar beet seeds (Højland and Pedersen, 1994). Sugar beet seeds may remain in the soil for ten years or more and still retain some germination capacity (OECD, 1993b; Brouwer *et al.*, 1976; Lysgaard, 1991). It is generally accepted that six year-old multigerm and four year-old monogerm sugar beet seed exhibit the same germination level of 70%. Eight-year-old sugar beet seeds have been shown to germinate at a level of 59% in laboratory conditions. These germination percentages depend of the quality of the seeds and of the conditions of germination. Thus *Beta vulgaris* has the ability to generate a viable seed bank (Højland and Pedersen, 1994). The seed-balls of *Beta* are resistant to salt water, and ocean currents can move propagules over relatively long distances. Above the high water line, strong winds distribute them over the shoreline, and sometimes even inland (Smart, 1992).

Since commercial sugar producing sugar beet is biennial and is harvested during the first year whilst still in the vegetative phase, sexual reproductive organs (floral parts) never develop. Varieties that tend to bolt in the first year of growth pose some problems and much effort has gone into developing currently cultivated varieties that limit bolting. When *Beta vulgaris* is planted for seed production, some seeds may remain on the field after harvesting the seed crop. Agricultural practices tend to limit those shoots.

#### **F. Isolation measures and distances**

As pollen is mainly wind-borne, large isolation distances are necessary to prevent pollination from sources other than the desired male parent. For commercial seed production, isolation distances

are very variable according to the country. In the literature, it varies from 1 to 3.2 (Campbell and Mast, 1971; Smith, 1980; Højland and Pedersen, 1994).

The OECD developed a scheme in order to homogenise the isolation distances (OECD Council Decision of 10<sup>th</sup> October 1988 (C [88] 66), appendix II).

## 2. Agronomic Practices

Sugar beets are cultivated all over Europe (including the former USSR) and in the USA (FAO Year book, 1992; Højland and Pedersen, 1994). Various biotypes of beet are found in cultivation throughout Europe (De Bock, 1986) and outside Europe in North Africa, Asia and in North and South America (FAO Yearbook, 1992).

In central Europe sugar beet is usually grown at altitudes below 400m. Climate affects both beet yield and sugar content. To produce high sugar content, sugar beet requires at least 170 growing days and high levels of sunlight (Brouwer *et al.*, 1976) but in Nordic countries this growing period is shorter (only 150 days). The crop also requires high amounts of moisture. Sugar beet crop fields are irrigated in regions with low precipitation. Sugar beet roots are slightly tolerant of acidic conditions, although soils with a pH of 7-8 are suitable.

Genetically monogerm seed is used almost exclusively in sugar beet cultivation except in China. Multigerm varieties are still used in Europe, South Africa, Near East and North America. Calibrated and pelleted seed is available. Calibrated seed has been mechanically separated. This process is relatively crude, resulting in fragments of quite different sizes. The monogerm seed for sugar beet on the market is almost exclusively in pellet form. Pellet seed is encased in a coat containing components used to control diseases and pests (Geissler, 1988). In terms of form and size, pelleted seed represents an extremely uniform seed type.

## 3. Centres of Origin/ Diversity, Geographic Distribution, Close Relatives and Their Geographical Distribution

### A. History of cultivated beet

Beet was a well-established vegetable in “classical” ancient Greece and Rome. The earliest documentation comes from eighth century B.C.E. Babylonia. Greek, Roman and Jewish literary sources provide clear information that in the first century BC the crop was represented by several leafy forms (chards). Cultivars with swollen roots appeared later. There are no archaeological records of *Beta vulgaris* from pre-classical times, and it is not known exactly when and where beet was domesticated. The wild forms from which the crop could have been derived are widely distributed over the Mediterranean basin and the Near East (Zohary and Hopf, 1994).

The first known description of beets are of foliage beets (or chards) by Aristotele (c. 350 BC), who described a red chard, and Theophrastus (c. 300 BC) who recognised two different beets, white and black, the colours referring to light and dark green appearance of the leaves. The use of roots of beet are referred to for both culinary and medicinal purposes by Roman writers (Fort-Lloyd and Williams, 1975).

Beet leaves were probably used as potherbs (herbs used in cooking) in prehistoric times. In the sixth to fourth century BC, the first cultivated forms were developed and used as salad vegetables (chards). Red fodder beet has been cultivated since the 15<sup>th</sup> century, and sugar beets only since the end of the 18<sup>th</sup> century (Frietema-De Vries, 1996).

*Beta vulgaris* L. ssp. *maritima*, wild sea beet, is regarded as the mother species of the *Beta* beets (fodder beet, sugar beet, beetroot, yellow beet, Swiss chard). It is indigenous to European coastal regions, particularly the Mediterranean. Beet spinach, convar. *cicla*, has been cultivated in the Mediterranean region since 2000 B.C. In Europe *B. vulgaris* species with distinctly swollen roots were cultivated in the Middle Ages. Central European types are presumed to be descended from those used in Arabian horticulture in Spain. These plants were taken to the Netherlands, where they were cultivated beginning in 1500, and then to the Palatinate region, later spreading throughout Germany as “Burgundy beet”. During the sixteenth and seventeenth centuries, red and yellow beets became increasingly common as salad vegetables. Fodder beet cultivation only began to increase during the course of the eighteenth century. The crop was introduced into the USA in 1800 where it became known as a garden beet. Sugar beet was introduced to North America around 1830 and to South America circa 1850 (Mansfeld, 1986).

In 1747, when the pharmacist Markgraf found that the sweet substance in beets was sucrose, efforts to extract sugar from beets began. At this time the sucrose content was 6.2%. Some forty years later in 1786, the breeder Achard selected from 23 local beet varieties a plant from the Halberstadt area for beet-sugar production. Koppy and Sohn selected the local variety “white Silesian Sugar beet.” This submerged-root variety became the mother type for all sugar beet varieties. A student of Markgraf built the first factory extracted the sugar from beet in 1801 (Campbell, 1976; OECD, 1993b) and produced the first “variety” White Silesian. In the following 70 years, selection produced a beet variety with sugar contents of 16%. Today’s sugar beet has a sugar content of 18-20%. In 1925, the global production of beet sugar represented 50% of the cane sugar production. By 1982, 30% of all sugar produced was from sugar beets.

## B. Origin

Sugar beet originates from the cultivated form of beet around the Mediterranean area; region A1 (Near East) (Pernès J., 1984). It is possible that all cultivated beets originated from *B. maritima* (Mc Farlane J.S., 1971).

## C. Close relatives and their geographic distribution

*Beta vulgaris* ssp. *maritima* (wild sea beet), is a common seashore plant of the coasts of Europe and Western Asia, and is perhaps a complex of closely related species. Primitive “superior” forms of this plant have been used as a leaf vegetable since prehistoric times and Root Beets, the ancestors of modern varieties, have been cultivated since the first century (De Rougemont, 1989).

*Beta vulgaris* ssp. *maritima* has spread from the centre of origin (Near East) to the coastal areas along the Mediterranean Sea to the Canary Islands, the Azores and along the Atlantic coast to Ireland and the southern parts of Scotland. It has been found in Belgium. It is rare in Holland and Germany (only known from the island Helgoland) and there is an isolated area of distribution in Denmark and at the Swedish Kattegat coast (Højland and Pedersen, 1994). It is not known in Switzerland. In Eastern Europe, it is found in Bulgaria and Romania. The area of distribution extends eastward to Iran, India, China and other Asian countries (Højland and Pedersen, 1994). Cultivated beet seed production areas are sometimes adjacent to sea beet populations (Bartsch *et al.*, 1999).

Ssp. *maritima* occupies a very narrow coastal niche between high tide level and 10 to 20 meters inland (Doney, 1992). Neither sugar beet nor sea beet is naturalised in habitats away from the coast (Højland and Pedersen, 1994). Ruderal beet from South-western France are very close to Mediterranean *Beta maritima*.

Distribution of the *Beta* species is shown in 1.18:

**Table 1.17 Distribution of the five *Beta* species present in Europe**

	<i>B. vulgaris</i> L.	<i>B. macrocarpa</i> Guss.	<i>B. patellaris</i> Moq.	<i>B. trigyna</i> Waldst. and Kit.	<i>B. nana</i> Boiss. and Heldr.
Albania	X				
Azores	X				
Belgium and Luxembourg	X				
Baleares	X				
Britain	X				
Bulgaria	X			X	
Corsica	X				
Crete	X				
Former Czechoslovakia				X	
Denmark	X				
Finland	X				
France	X	X		X	
Germany	X				
Greece	X	X			X
Ireland	X				
Italy	X	X		X	
Former Yugoslavia	X			X	
Netherlands	X				
Portugal	X	X			
Romania				X	
Former Russia	South western part			Crimea	
Sardinia	X				
Spain	X	X	X		
Sweden	X				
Switzerland	X				
Turkey	X				

(See Tables 2.16 and 2.17. for the general distribution of *Beta* species)

*Legend:* the presence of an X indicates the presence of the *Beta* species in the country.

#### 4. Reproductive Biology

##### A. Flower morphology

Flowers of *Beta vulgaris* ssp *vulgaris* are located on the terminal portions of the main axis and on lateral branches subtended from this. Flowers are sessile and occur singly or in clusters of two to eight (Smith, 1980).

Flowers are perfect and consists of a tricarpellate pistil surrounded by five stamens and a perianth of five narrow sepals (Smith, 1980). The flowers, solitary or in clusters of 2-8, are rarely self-pollinating (Free, 1970). The flower has a raised ovary with three or four secure stigmata. Three leaves are fused together into a single gynoeccium to form the ovary. The seed arrangement is campylotropous.

## B. Compatibility

Beet is a strongly self-incompatible plant (the stigma is not fully mature when the flower opens). Plants set few or no seeds at all when isolated (OECD, 1993b; Smith, 1980; Valdeyron, 1984). Self-fertilising plants exist in nearly every beet population (Barocka, 1985). Their frequency is lower among tetraploids than among diploids. Selecting for the diploid characteristic can significantly increase the potential for self-fertilisation.

The incompatibility system is genetically controlled by a complex gametophytic system governed by at least four loci, each with a number of alleles, and is further influenced by modifying genes. The *Beta* populations found on shores around the North Sea are largely self-incompatible (Dale and Ford-Lloyd, 1985). Mediterranean *B. maritima* populations are highly self-fertile due to specific gene for self-fertility (Fédération Internationale du commerce des Semences indication).

Sugar beet and sea beet (*Beta vulgaris* ssp. *maritima*) are both protandrous, self-incompatible. Sugar beet is an allogamous species, pollinated by wind and occasionally by insects, the former being the most important. Some cross-pollinations are also achieved by thrips and syrphids (Free J.B. and al., 1975; Valdeyron, 1984).

## C. Formation of reproductive parts

The reproductive phase of sugar beet occurs during the second growing season. During the generative growth phase, following vernalisation, the internodes become extended into shoot bearing leaves that are ellipsoid to shape. Leaf axils have buds out of which the flower bearing shoots, panicles, emerge. Often only a single, very strong, unbranched flower shoot develops, although in some instances many flowering stems grow and form an extensive bush. Flowering stems are upright, up to two meters in height, green and coarsely furrowed. The secondary shoots may stretch upward or may hang down.

Temperatures above 21°C favour vegetative growth and temperatures between 4 and 13°C favour initiation of the reproductive phase. Most commercial cultivars of sugar beet require 90 to 110 days of exposure to inductive temperatures for initiation of reproductive development (Smith, 1980). With cultivars that bolt easily, comparatively short exposure to cool temperatures is adequate to induce plants to flower. Cultivars that do not bolt readily require longer periods of cold temperatures for floral induction (Smith, 1980). Photoperiod (short days) has also a strong effect on the vernalisation process.

Flower formation commences on the top shoot and flowers mature from the base upwards, growth of the secondary shoots following afterwards. Individual flowers of the cluster, made up of five thin inwardly curved perianth leaves and five stamens, do not flower synchronously. They are joined by gland-like tissue at the base, and this excretes large amounts of honey. The middle flower of the cluster blooms first followed by the surrounding flowers. Protandry exists at the morphological level. The sugar beet plant flowers for duration of approximately four weeks.

Depending on the cultivar and environmental conditions, mature flowers begin anthesis about 5 to 6 weeks after initiation of reproductive growth and continue for several weeks. Flower opening begins at the base of each stem and continues upward as the stem elongates. Flower open mostly in the morning, but continue throughout the day (Smith, 1980). Stigmas may remain receptive for more than two weeks allowing a good chance for wind blown pollen to effect fertilisation depending on weather conditions (Crane and Walker, 1984). The flowering period for sugar beet in Central European climatic is between June and August (Barocka, 1985).

### D. Pollen

Pollen grains are round and have numerous indentations in their wartlike exines. The number of pollen grains per anther is estimated at 17 000. This would correspond to 85 000 grains per flower and, given 10 000 flowers per bush, almost one billion per plant (1 ha sugar beet with circa 25 000 seed plants produced approximately 25 trillion pollen grains) (Schneider, 1942). The pollen ability of survival is limited to maximum 24 hours according to the German experts. This depends on the environmental conditions, especially moisture.

The genus *Beta* also displays pollen sterility. Nuclear male sterility is under the control nuclear genes restoring male fertility (although cultivated and wild beet may have different [Owen, 1945; Boutin *et al.*, 1988 and 1987]). The mechanism can also be cytoplasmic (Owen, 1945, 1952).

### E. Pollen dispersal

Pollen is transported primarily in air currents. Insects (honeybees, bees, thrips) play a lesser role in pollen movement. Honeybees may increase seed yields, but seem to visit plants only if no other pollen is available. A study of insect pollination of sugar beet seed crops revealed that most of the visiting insects (129 species) carried sugar beet pollen grains and probably contributed to cross-pollination (Free *et al.*, 1975). Because tetraploid plants produce fewer and larger pollen grains than diploid plants, and the pollen is less readily released by the anthers, insect pollination is probably more important for hybrids whose pollen donor is tetraploid than for pure seed crops. This is especially so when relative humidity is high, and little pollen is transported by air movement, yet insect activity is unaffected (Free *et al.*, 1975).

Wind-borne pollen can be distributed horizontally at least 4,500 m and has been observed at a height of 5,000 m (Archimowitsch A., 1949). Smith (1980) observed pollen drift of up to 5 km from the originating field, and Gliddon (in Harding and Harris, 1994) assumed that the airborne pollen movement can occur at distances up to 8 km.

### F. Pollination

Tetraploid plants release their pollen somewhat later in the day than diploid plants and there is a period in the morning when the stigmata of the male-sterile plants are receptive, but when the pollen donors are not producing an effective pollen cloud. It is during this period that the male-sterile mother plants are most receptive to contamination from background pollen released by wild and weedy forms of diploid *B. vulgaris*, many of which, in continental Europe, are annuals or near annuals. This leads to the production of weedy forms of beets. This contamination is a potentially serious weed problem (Scott and Longden, 1970; Longden, 1976; Hornsey and Arnold, 1979).

### G. Seed

The fruit of the sugar beet is a capsule and seed is imbedded in a hollow that remains closed by a small lid that springs open during germination although the seed remains firmly attached to the pericarp. The seed is circular, approximately 1-2 mm in diameter, and has a kidney shaped indentation caused by a small, beak-shaped root. According to Martin and Leonard (1976) and Benjamin and Bell (1985) flowering sugar beet can produce 200 million seeds per hectare. What is generally referred to, as the beet seed is in fact a cluster-like multiple fruit. Sugar beet seed normally consist of a seed-ball formed by two to four true seeds (OCDE, 1993b). 1000 clusters weigh between 10-40 g.

The ovaries are enclosed by the common receptacle of the flower cluster (Smith, 1980). They form a hard and irregular dry body, the so-called seed ball, which usually contains one to four seeds.

A plant with monogerm seed borne in separate flowers was found in the United States in 1948 (Martin and Leonard, 1976). Before the genetically monogerm seed was found, they were made mechanically by grinding seed clusters (Højland and Pedersen, 1994). The characteristic of monocarpy is recessive and determined by one gene. Today most cultivated sugar beet varieties are monogerm and whose seedlings need not be thinned after sowing and germination (Barocka *et al.*, 1968; Winner, 1981).

## H. Seed dispersal

In wild beet a large proportion of mature seeds produced by the end of the flowering season are shed in the immediate vicinity of the maternal plant. Some of the seeds, however, may be dispersed over greater distances. The major agent of long distance dispersal of the multigerm seedballs of ssp. *maritima* is believed to be tidal movements of the water (Dale and Ford-Lloyd, 1985).

Sugar beet seed do not shatter as easily as some wild *Beta* species, which may drop their seeds as they ripen. Seeds falling on the ground do not usually germinate in the same season, partly because of the presence of germination inhibitors, partly because of poor seed-soil contact (OECD, 1993b; Letscghert, 1993).

## 5. Cross

### A. Intraspecific

The six *Beta vulgaris* subspecies are interfertile although individually they are self-incompatible. The cultivated species *Beta vulgaris* ssp. *vulgaris* is characterised by a great varietal diversity. In addition to sugar and fodder beets, there also exist leaf spinach beet, Swiss chard, and red beet (see Table 1.15). All varieties may cross with one another, a characteristic that must be taken into account in seed production.

Sugar beet and sea-beet (*Beta vulgaris* ssp. *maritima*) hybridise freely and hybrids are spontaneously formed in the wild and in seed-production fields (Bartsch *et al.*, 1999). Such hybrids are fertile and do not demonstrate incompatibility at the chromosome level (Evans and Weir, 1981). The most important precondition for hybridisation in natural habitats is the existence of spatially overlapping populations and flowering periods. *Beta vulgaris* ssp. *vulgaris* and *Beta vulgaris* ssp. *maritima*, which hybridise the most frequently in nature, share a common flowering period from May to September. They can occur in the same areas, as ssp. *maritima* is distributed along the Atlantic coastal region (Hanf, 1990). As already noted, these hybrids are an important source of so-called weed beets among the cultivated types of West Europe and North America (Hornsey and Arnold, 1979; Evans and Weir, 1981).

### B. Interspecific

#### *Beta section*

*Beta vulgaris* belongs to the section *Beta* (syn. *vulgare*) together with *B. maritima*, *B. macrocarpa*, *B. patula* and *B. vulgaris* ssp. *adanensis*, the wild species of the cultivated beet (Valdeyron, 1984; Smart, 1992). All these species are cross compatible (Smith, 1980; Bartsch *et al.*, 1999). The hybrids are vigorous and fertile and do not show incompatibility at the chromosome level

(Geyt Van *et al.*, 1990). However, hybrids between beet and *B. macrocarpa* are rare due to differing flowering times of the parental species (Mc Farlane, 1975). Wild species of the section *Vulgares* (*B. vulgare* ssp. *maritima*, *B. orientalis*) have been used in beet breeding (Geyt Van *et al.*, 1990).

Abe *et al.* (1984) observed that hybridisation between *B. macrocarpa* and *B. vulgaris*, and between *B. maritima* and *B. atripicifolia* result in a certain degree of pollen sterility and seed abortion of the F<sub>1</sub> generation. Lange and De Bock (1989) produced triploid and tetraploid hybrids between tetraploid *B. macrocarpa* and diploid and tetraploid types of *B. vulgaris*. The triploid descendants were sterile although the tetraploid descendants exhibit a better fertility. The F<sub>2</sub> is partially fertile. A number of researchers have reported successful crosses between *B. vulgaris* and species of section *Corollinae* (reference in Geyt *et al.*, 1990).

#### **Corollinae section**

Artificial hybrids can be produced with the species of the *Corollinae* section, but such hybrids are mostly sterile and only set a few seeds when backcrossed to sugar beet (OECD, 1993b).

No evidence of interfertility has been found between the cultivated beet and the Caucasian beet (*Beta trigyna*).

#### **Procumbentes section**

Artificial hybrids with members of the *Procumbentes* section usually die at the seedling stage. They can be saved by grafting onto sugar beet, and they then develop into vigorous plants. These hybrids are also almost completely sterile and set few seeds upon backcrossing (OECD, 1993b).

The majority of *Beta vulgaris* ssp. can be crossed with wild species of the section *Procumbentes*. Jung and Löptien (1986) achieved crosses between sugar beet and *B. procumbens*, *B. webbiana* and *B. patellares*. The F<sub>1</sub> hybrids were backcross with *B. vulgaris* (See also Højland and Pedersen, 1994) to establish a complete set of monosomic addition lines (2n = 19).

#### **Nanae section**

No hybrids between sugar beet and *B. nana* are known (OCDE, 1993b).

### **C. Introgression**

There is extensive evidence of hybridisation in the wild between, and introgression from wild beet to cultivated sugar beet and vice versa.

### **D. Interactions with other organisms**

The sugar beet leaves contain oxalic acid which can cause problems if fresh, unwilted sugar beet tops are used as cattle feed (OECD, 1993b).

Interactions between *Beta vulgaris* ssp. *vulgaris* with common disease organisms and pests are shown in Appendix I.

## **6. Weed Characteristics and Weediness**

Sugar beet may become a weed through the roots or crowns remaining in the field after harvest. These “volunteer” plants, if left, will flower and produce seed. The offspring of these plants is normally controlled by herbicide treatment or other means in the following crops.

Cultivated beet may possibly run wild but it is difficult to distinguish between cultivated beets and the weed beet. Beet is often found outside cultivation but there is no indication of such plants establishing in the wild (Frietema, 1996).

Sugar beet bolters could produce enough viable seeds to become a weed problem. This “weed beet” is seldom found in winter cereals, sometimes in spring cereals, especially where poor establishment has occurred, but is relatively common in potato and pea crops. This is because of the limited selection of herbicides that can be used, and because of the similar timing of cultivation techniques. It is estimated that one field in four in England will have viable beet seed in the top soil (Højland and Pedersen, 1994).

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## APPENDIX

### Interactions: Common Diseases and Pests of *Beta vulgaris*

#### Viral diseases

##### *Beet yellows (BYV and BMV)*

Beta virus 4 causes beet yellow. The disease is transmitted by aphids (primarily by *Myzus persicae* and *Aphis fabae*). Leaf yellowing is the principle symptom. The disease is of considerable economic importance as it leads to significant reduction in beet and sugar yield, particularly in regions where the transmitting aphids appear early (Brouwer *et al.*, 1976; Geissler, 1988; Heitefuss *et al.*, 1993).

##### *Sugar beet mosaic virus*

Beta virus 2 is responsible for this disease which is transmitted by various aphids. Symptoms include whitish or light green mosaic-like patterns on the inner part of young leaves. Sugar beet mosaic virus rarely causes severe economic damage (Brouwer *et al.*, 1976; Geissler, 1988).

##### *Beet-leaf curl*

Beet leaf curl is caused by Beta virus 3. The disease is transmitted by the beet leaf bug *Piesma quadrata*. Symptoms are leaf curling and the mosaic-like lightening of young leaves. Leaf veins swell. Severe epidemics can lead to yield reduction (Brouwer *et al.*, 1976; Geissler, 1988; Heitefuss *et al.*, 1993).

##### *Rhizomania*

Beet necrotic yellow vein virus (BNYVV) is responsible for rhizomania. The soil fungus *Polymyxa betae* transmits BNYVV. Infected plants display stunted growth, the leaves are smaller, light green, and stand straight up. A strong dense, root network is characteristic of the disease. The disease has been observed in Germany since the mid-1970s. BNYVV represents a serious threat to stricken crops (yield reduction of up to 50%) (Brouwer *et al.*, 1976; Barocka, 1985; Geissler, 1988; Heitefuss *et al.*, 1993). But it is spreading and is present all over Europe except Ireland and Denmark.

#### Bacterial diseases

##### *Beet leaf spot*

*Pseudomonas syringae* is responsible for this bacterial disease known as beet leaf spot. The bacteria enter the beet leaf through the stomata and wounds in the leaf. Brown to black spots of various sizes develop. Infected tissue breaks off. The disease has occurred in central and western Europe with increased frequency in recent years, but has not had significant economic effects (Heitefuss *et al.*, 1993).

*Crown gall*

The disease is caused by *Agrobacterium tumefaciens*. Crown gall can be identified by abnormal tissue growth on the body of the beet near the soil surface. The bacteria enter the beet through small wounds. Damage from crown gall is negligible from an economic point of view (Brouwer *et al.*, 1976).

*Common beet scab*

*Actinomyces scabies* induces the disease. The scab colours the rind of the beet blackish brown. Infected beets lignify severely. Beet scab does not represent a significant economic threat to beet cultivation (Brouwer *et al.*, 1976).

**Fungal diseases***Root rot and secondary root rot*

The disease is transmitted by fungi contaminating both seeds and soil. *Phoma betae* is transmitted on the beet seed, and infection after cold-weather sowing can cause significant damage during seedling emergence. *Pythium* species and *Aphanomyces* infect the seedling by means of motile zoospores. Damp conditions and cool temperatures increase the likelihood of *Pythium* infection. *Aphanomyces* requires higher temperatures for infection. Symptoms are often discernible on secondary roots. Many fungus-stricken seedlings do not emerge and those that do remain retarded in their development. If the plant survives the disease, growth remains stunted due to secondary root damage and rotting of the root tips. (Brouwer *et al.*, 1976; Geissler, 1988; Heitefuss *et al.*, 1993).

*Beet leaf spot diseases*

In warmer areas with high rainfall, beet leaf spot is the fungus that causes the most economic damage in sugar beet production. It is induced by a variety of fungi. The disease is caused by *Cercospora beticola*, for instance. The fungi can be transmitted on beet seed, but can also move on dead beet leaves or beet tops. The fungus' spores develop at temperatures at or above 17°C (optimum: 27°C) and require high air humidity. Wind and rain carry the spores onto the leaves of the beet plant where they germinate and enter the plant through the stomata. The first spots develop a few days thereafter. The fungus then sporulates once again, leading to renewed infection and the further spread of the disease throughout the crop. Crop damage can be observed in late June with old leaves showing the first symptoms. Initially, 2-3 mm round, reddish spots develop which later turn grey in the centre. As the disease progresses, the spots enlarge and merge and the leaves dry out. If the entire crown dies, the plant responds with new growth.

Beet leaf spot can also be induced by *Phoma betae*. The optimal temperature for development of this disease is 20°C. Diseased sugar beets grown for seed can result in a significant infection of the seed. Symptoms include round leaf spots with lighter centres and concentric dark and light rings. The disease is generally not an economically significant problem.

*Ramularia beticola* enters the beet through the stomata. Optimal infection temperatures are 18-20°C with a relative humidity of over 95%. After infection, 1 cm leaf grey to brownish spots develop. The disease is primarily of importance in seed beet production.

### *Powdery mildew*

The disease is caused by *Erysiphe betae*. The fungus develops well during dry weather with temperatures around 20°C. Initial infection is induced by spores that may have travelled over long distances. Signs of damage can be observed beginning in mid July. A white, powdery layer develops on the upper side of older leaves and quickly covers the entire leaf. The disease has been identified in central Europe since the early 1970s. Crops infected early can be severely damaged (Brouwer *et al.*, 1976; Geissler, 1988; Heitefuss *et al.*, 1993).

### *Downy mildew*

*Peronospora forimosa* requires high air humidity and temperatures below 15°C. A grey mildew layer covers upper and lower leaf surfaces. The disease is generally insignificant in central Europe (Brouwer *et al.*, 1976).

### *Other fungal diseases*

Leaf scorch (*Helicobasidium purpureum*), beet rust (*Uromyces betae*), violet root rot (*Helicobasidium purpureum*) and sclerotinia rot (*Sclerotinia sclerotiorum*, *S. fuckeliana*) may infect sugar beet, but generally do not cause significant biological or economic damage in central Europe (Brouwer *et al.*, 1976).

## **Animal pests**

### *“Finger” beetle*

The beetle (*Clivina fossar*) is about 6 mm long and has a small, nearly cylindrical body. Its colouring is reddish brown and it inhabits upper soil layers if sufficient moisture is available. From here the beetle attacks young plants. Occasionally, it causes extensive damage in sugar beet, particularly when fields are sown early and are weed-free after comprehensive herbicide application (Heitefuss *et al.*, 1993).

### *Pygmy mangold beetle*

The beetle (*Atomaria linearis*) grow to a length of 1.2-1.7 mm. It is dark in colour and spends the winter in plant remains on field borders and in the ground. The pygmy beetle migrates into beet fields in the spring. The hypocotyls of afflicted plants reveal dark, pin-head-sized bite marks. Plants may die in instances of extreme beetle damage. Considerable economic damage can result when beet crops are planted in succession (Brouwer *et al.*, 1976; Geissler, 1988; Heitefuss *et al.*, 1993).

### *Beet cyst nematode*

*Heterodera schachtii* is a “thread worm”. In addition to beets, cruciferous plants are among the beet cyst nematodes primary victims. Also known as the “beet eelworm”, beet cyst nematodes reach a length of 1.5 mm. Approximately 250 eggs per cyst develop into larvae. A portion of the larvae hatch in the summer and migrate into the roots of the beet. Sexually mature nematodes develop after they have absorbed enough nutrients. The swelling of the females causes the root tissue to break open. After mating, the female hardens and develops into a cyst. Eggs and larvae remain viable in this phase for years. Infested crops mature irregularly, wilt, turn yellow, and die. Plants also develop an unusual number of secondary roots and a so-called “root beard” displaying many cysts. Severely infested soil can result in significant yield reduction. Beet cyst nematodes are considered to be partly responsible

for beet sickness affecting soil (Brouwer *et al.*, 1976; Loptien, 1984; Barocka, 1985; Geissler, 1988; Heitefuss *et al.*, 1993).

#### *Collembolans*

Colembolans (*Onychiurus armatus*) are white, 1-2 mm in length, and live underground, usually using plant detritus in the soil as a food source. Lacking these sources of nutrients, the pest attacks the roots of sugar beet seedlings. Seeds and hypocotyls may be damaged. Plants may die in cases of severe infestation. Colembolans do not account for significant economic losses in sugar beet cultivation (Heitefuss *et al.*, 1993).

#### *Wire worm*

The brown beetle (*Agriotes ssp.*) is 6-12 mm long. Eggs are laid in the summer and the larvae develop into adults over the course of 3-5 years. They can cause feeding-related damage beginning in their second year of development. The roots of young beets may be partially or completely eaten. The plants wilt and die. Wire worm infestation can result in considerable yield reductions in years with high precipitation or when new land is cultivated (Brouwer *et al.*, 1976; Heitefuss *et al.*, 1993).

#### *Millipede*

Millipedes (*Blaniulus guttulatus*) are light-coloured, approximately 1 mm in diameter, and grow to a length of 20 mm. They have a life span of two years. They occasionally cause feeding-related damage to seeds and seedlings.

#### *Beet-leaf fly*

*Pegomya betae* overwinters as a pupa in the soil. The larvae bore into leaf tissue within 4-10 days after hatching. The larvae exit the leaves after feeding for 2-3 weeks and pupate in the soil. Two to three generations develop each year, but only the first is relevant as a pest. Leaf tissue damaged between the top and bottom sides dries, splits apart, and eventually dies. Crops that are afflicted early in the season may be severely damaged. Damage rarely occurs after the beet has reached the 6-leaf stage. Economic damage due to beet-leaf flies has been on the decline in recent years (Brouwer *et al.*, 1976; Heitefuss *et al.*, 1993).

#### *Green peach aphid*

*Myzus persicae* spends the winter as an egg in peach (*prunus persica*) and cherry (*Prunus* species) trees. In the spring, the mother aphid hatches and produces wingless aphids. Winged aphids develop beginning in May and these migrate to beet. Here they reproduce asexually for a number of generations. Infested plants can be recognised by their slightly discoloured leaves. The green peach aphid causes significant economic damage as a carrier of viruses responsible for beet yellows (Heitefuss *et al.*, 1993).

#### *Blackfly*

*Aphis fabae* spends the winter in egg form in the European Euonymous and the snowball tree (*Viburnum opulus*). Its development is similar to that of the green peach aphid. Infested plants display rolled leaves; young leaves are strongly curled. Severe damage and yield reduction is caused primarily by the sucking activity of the blackfly, although the pest is also a transmitter of viruses (Brouwer *et al.*, 1976; Geissler, 1988; Heitefuss *et al.*, 1993).

*Field slug*

*Deroceras reticulum* attains a length of 50-65 mm. The slugs have a scale-shaped shell and are yellow-white or red-brown in colour. Field slugs damage almost all cultivated plants. They spend the winter as eggs or slugs. Mild winters allow them to multiply more quickly. They eat away at the leaves until only the more sturdy veins remain. The damage caused by field slugs has increased in recent years, and not only with respect to beet cultivation. Rape cultivation, rotational crops, and fallow fields have led to a general increase in the field slug's impact on agriculture (Heitefuss *et al.*, 1993).

*Common wood mouse*

The common wood mouse (*Apodemus sylvaticus*) is grey with a brown-grey to brown-red stomach. It has a short tail and large eyes and ears. Field woods, wood borders, fields and gardens are its habitat. The common wood mouse only gained significance as a pest with the introduction of pelleted beet seed. The mouse causes damage by digging up seeds along the drilled rows, cracking them, and eating the seedlings. This pest repeatedly causes severe damage in some areas (Heitefuss *et al.*, 1993).

Some other herbivores attacks the beet:

*Agrosetis segetum*: roots

*Blitophaga linearis*: leaves of small plants

*Calocoris norvegicus*: leaves

*Discestra trifolii*: leaves

*Ditylenchus dipsaci*: stems and leaves

*Pegonya hyoscyami*: leaves

*Thrips angusticeps*: leaves and stems of small plants.

SECTION 9  
SUNFLOWER (*HELIANTHUS ANNUUS* L.)

**1. Taxonomy of the Genus *Helianthus*, Natural Habitat and Origins of the Cultivated Sunflower**

**A. Taxonomy of the genus *Helianthus***

The sunflower belongs to the genus *Helianthus* in the Composite family (Asterales order), which includes species with very diverse morphologies (herbs, shrubs, lianas, etc.). The genus *Helianthus* belongs to the Heliantheae tribe. This includes approximately 50 species originating in North and Central America.

The basis for the botanical classification of the genus *Helianthus* was proposed by Heiser *et al.* (1969) and refined subsequently using new phenological, cladistic and biosystematic methods, (Robinson, 1979; Anashchenko, 1974, 1979; Schilling and Heiser, 1981) or molecular markers (Sossey-Alaoui *et al.*, 1998). This approach splits *Helianthus* into four sections: *Helianthus*, *Agrestes*, *Ciliarens* and *Atrorubens*. This classification is set out in Table 1.18.

*Section Helianthus*

This section comprises 12 species, including *H. annuus*, the cultivated sunflower. These species, which are diploid ( $2n = 34$ ), are interfertile and annual in almost all cases. For the majority, the natural distribution is central and western North America. They are generally well adapted to dry or even arid areas and sandy soils. The widespread *H. annuus* L. species includes (Heiser *et al.*, 1969) plants cultivated for seed or fodder referred to as *H. annuus* var. *macrocarpus* (D.C.), or cultivated for ornament (*H. annuus* subsp. *annuus*), and uncultivated wild and weedy plants (*H. annuus* subsp. *lenticularis*, *H. annuus* subsp. *Texanus*, etc.).

Leaves of these species are usually alternate, ovoid and with a long petiole. Flower heads, or capitula, consist of tubular and ligulate florets, which may be deep purple, red or yellow.

*Section Agrestes*

This section includes only the annual species *H. agrestis* ( $2n = 34$ ), characterised by reddish coloured tubular flowers, yellow styles and glabrous stems bearing leaves that are generally opposite and lanceolate (Bonjean, 1993). Its self-compatibility makes it different from other species (Heiser *et al.*, 1969). It is well suited to the moist soils of central Florida and Georgia.

*Section Ciliarens*

This section includes six perennial species of small size originating in Mexico and the western United States. They are genetically quite distinct from the species in the other sections.

A distinction is made between two series in this section: *Ciliarens* and *Pumili*. The first includes three species possessing powerful secondary root systems, making them redoubtable adventives. Their leaves are

usually opposite, bluish in colour, virtually glabrous and with very short petioles. All the species in this series are diploid, with the exception of *H. ciliaris*, which includes tetraploid ( $2n = 68$ ) and hexaploid ( $2n=102$ ) populations.

The species in the *Pumili* series have opposite hairy leaves, growing from buds which appear at the crowns of the old roots. They are all diploid ( $2n = 34$ ).

#### *Section Atrorubens*

This section includes thirty species divided arbitrarily into four series, including one cultivated species, the Jerusalem artichoke (*H. tuberosus* L.). Hybridisation between these species and their polyploid forms can make it difficult to classify them precisely.

The natural distribution of these species is the eastern and central United States, with the exception of *H. nuttallii* and *H. californicus*, which are found only in the West.

The *Corona-solis* series contains large species with tuberiform or rhizomatous roots. In some cases, leaves are alternate, large and numerous (*H. giganteus*, *H. grossesserratus*, *H. nuttallii*, etc.). Others have opposite, lanceolate leaves with three main veins (*H. divaricatus*, *H. mollis*). Seven species are diploid. *H. strumosus* and *H. decapetalus* can be found in tetraploid and hexaploid forms. *H. hirsutus* is tetraploid. Six species are hexaploid.

The *Atrorubentes* series comprises four perennial species from the southwest of the United States. Usually without rhizomes and with fibrous roots, they go through a very marked rosette stage. These species were formerly included in the *Divaricati* series in Heiser's classification.

The *Microcephali* series includes four species which have a small capitulum and roots that are fibrous or perhaps slightly rhizomatous. Their stems may be covered with wax and bear leaves that are alternate in most cases. A fifth species, Porter's sunflower (*H. porteri*) has been transferred from the genus *Viguiera* to the genus *Helianthus* (Yates and Heiser, 1979). This is an annual species found only in Georgia.

The species in the *Angustifolii* series are all diploid and located essentially in the southwestern United States. Their stems are hairy and the leaves usually alternate, lanceolate and with a leaf blade that may curl in toward the inside surface. Their roots are fibrous, thick or with rhizomes.

Table 1.18 Classification of the genus *Helianthus* (Seiler and Rieseberg, 1997)

Section	Series	Species	Ploidy
<i>Helianthus</i>	-	<i>H. annuus</i> L. *	2n = 34
		<i>H. anomalous</i> Blake *	2n = 34
		<i>H. argophyllus</i> T. & G. *	2n = 34
		<i>H. bolanderi</i> A. Gray *	2n = 34
		<i>H. debilis</i> Nutt. *	2n = 34
		<i>H. deserticola</i> Heiser *	2n = 34
		<i>H. exilis</i> A. Gray *	2n = 34
		<i>H. neglectus</i> Heiser *	2n = 34
		<i>H. niveus</i> (Benth.) Brandegee *	2n = 34
		<i>H. paradoxus</i> Heiser *	2n = 34
		<i>H. petiolaris</i> Nutt. *	2n = 34
		<i>H. praecox</i> Engelm. & A. Gray *	2n = 34
<i>Agrestes</i>	-	<i>H. agrestis</i> Pollard *	2n = 34
<i>Ciliaries</i>	<i>Ciliaries</i>	<i>H. arizonensis</i> R. Jackson	2n = 34
		<i>H. ciliaris</i> DC.	2n = 68, 102
		<i>H. laciniatus</i> A. Gray	2n = 34
<i>Ciliaries</i>	<i>Pumili</i>	<i>H. cusickii</i> A. Gray	2n = 34
		<i>H. gracilentus</i> A. Gray	2n = 34
		<i>H. pumilus</i> Nutt.	2n = 34
<i>Atrorubens</i>	<i>Corona-solis</i>	<i>H. californicus</i> DC.	2n = 102
		<i>H. decapetalus</i> L.	2n = 68, 102
		<i>H. divaricatus</i> L.	2n = 34
		<i>H. eggertii</i> Small	2n = 102
		<i>H. giganteus</i> L.	2n = 34
		<i>H. grosseserratus</i> Martens	2n = 34
		<i>H. hirsutus</i> Raf.	2n = 68
		<i>H. maximiliani</i> Schrader	2n = 34
		<i>H. mollis</i> Lam.	2n = 34
		<i>H. nuttallii</i> T. & G.	2n = 34
		<i>H. resinosus</i> Small	2n = 102
		<i>H. salicifolius</i> Dietr.	2n = 34
		<i>H. schweinitzii</i> T. & G.	2n = 102
		<i>H. strumosus</i> L.	2n = 68, 102
		<i>H. tuberosus</i> L.	2n = 102
<i>Atrorubens</i>	<i>Microcephali</i>	<i>H. glaucophyllus</i> Smith	2n = 34
		<i>H. laevigatus</i> T. & G.	2n = 68
		<i>H. microcephalus</i> T. & G.	2n = 34
		<i>H. smithii</i> Heiser	2n = 68
<i>Atrorubens</i>	<i>Atrorubentes</i>	<i>H. atrorubens</i> L.	2n = 34
		<i>H. occidentalis</i> Riddell	2n = 34
		<i>H. pauciflorus</i> Nutt. (synonym <i>H. rigidus</i> Cass.)	2n = 102
		<i>H. silphoides</i> Nutt.	2n = 34
<i>Atrorubens</i>	<i>Angustifolii</i>	<i>H. angustifolius</i> L.	2n = 34
		<i>H. carnosus</i> Small	2n = 34
		<i>H. floricornis</i> A. Gray ex Chapman	2n = 34
		<i>H. heterophyllus</i> Nutt.	2n = 34
		<i>H. longifolius</i> Pursh	2n = 34
		<i>H. radula</i> (Pursh) T. & G.	2n = 34
		<i>H. simulans</i> E.E. Wats.	2n = 34

\* annual species. The others are perennial.

**B. The natural distribution of sunflowers (*Helianthus annuus* L.)**

The species *H. annuus* comes originally from North America. It is the most diverse North American sunflower species not only in terms of its geographical distribution, but also with respect to its morphology and environmental and physiological adaptation (Seiler, 1984).

It is found at altitudes between sea level and 3,000 metres in areas with a range of different rainfall characteristics, but essentially in the western two-thirds of the United States, southern Canada and northern Mexico. It is usually found in open habitats already disturbed by human activity (Bonjean, 1993).

At maturity, these plants present a high degree of phenotypic variation: their size may vary from less than a metre to more than four metres. They may or may not be branched and may present varying degrees of hairiness. Their leaves, which are generally oval to cordate in shape, are alternate and petiolate, with a size in the range 5 cm to 35 cm wide and 10 cm to 50 cm long. The capitulum is at least 1.5 cm deep and supports relatively broad bracts which may be oval or lanceolate; it is rarely glabrous on the dorsal surface and is usually ciliate at the edges.

The ligulate flowers are approximately 25 mm in length and sterile. There are at least seventeen of these. The tubular flowers are shorter and have corollas with lobes that are purple, reddish or yellow in colour. The achenes are 3 mm to 15 mm in length and are found in a range of colours (for example white, black, black with white stripes, and brown).

In the natural state, the flowering of wild *H. annuus* is a lengthy process involving each capitulum in turn, and lasts from late July to early October. The species is diploid ( $2n = 34$ ) and hybridises naturally with many other sunflowers.

Wild populations are usually strictly self-incompatible and markedly allogamous, cross pollination being obligate. They are pollinated by insects, first and foremost by bees. Cultivated forms of sunflower generally allow a higher degree of self-compatibility.

### **C. The origins of the cultivated sunflower**

The cultivated sunflower probably comes originally from the western United States. It is certainly the case that wild sunflower seeds were a food resource for Native American populations living in this geographical region.

According to Heiser (1985), the most probable hypothesis as to the domestication of the sunflower is that it was an adventive found at the edges of Native American encampments. Inhabitants of these encampments gathered the achenes of wild sunflowers. In this way, the plant was carried from western to central North America. Based on this hypothesis, it was domesticated there and then introduced in the same period into the eastern and southern parts of the United States.

The fact that cultivated sunflower achenes have been found in several archaeological sites in the eastern and central United States, whereas archaeological digs in the southwest of the country and in Mexico have brought to light only the achenes of wild sunflowers, is one of the strongest pieces of evidence for Heiser's hypothesis.

Using isozyme systems and chloroplasmic DNA, Rieseberg and Seiler (1990) have been able to provide proof at the molecular level that the cultivated sunflower apparently derived from a virtually unique or adventive form of sunflower, which probably grew originally in the central United States.

The cultivated sunflower was introduced into Europe in the late 16<sup>th</sup> century, probably by Spanish sailors. It was initially grown as an ornamental. No mention is found of its advantages as an oilseed plant before the 18<sup>th</sup> century, and the sunflower has since seen major genetic advances. It was in Russia that the first such improvements were made to develop single headed, shorter, earlier flowering plants and to increase the oil content of the seeds. More recently, strains with high oleic acid content were developed by Soldatov (1976). For these reasons, Russia is considered as a secondary domestication centre for sunflower.

The cultivated sunflower presents a narrow range of genetic variability, notably with regard to certain agronomic and technical characteristics such as standability and oil content. This is all the more true of hybrids created during the last decade (Bonjean, 1993). Conversely, the wild species of the genus *Helianthus* present a high degree of genetic variability, the exploitation of which has enabled enormous progress to be made in the creation of varieties, especially with regard to increased oil content, as well as resistance to disease, insects and dry conditions (Leclercq *et al.*, 1970; Krauter *et al.*, 1991; Miller *et al.*, 1992; Serieys, 1984; Serieys, 1997). Interspecific hybridisation also enabled identification of many new sources of male cytoplasmic sterility (Serieys, 1999). Partial hybridisation mechanisms frequently observed between perennial *Helianthus* and cultivated sunflower (Faure *et al.*, 2002), represent another way for controlled introgression (Faure *et al.*, 2002).

## 2. The Botanical Characteristics of the Cultivated Sunflower

The principal morphological and physiological characteristics of the sunflower, such as height, diameter of the capitulum, duration of the growing cycle, size of seeds and oil content, are all highly dependent on the soil climate in which it is cultivated (Merrien, 1986).

### *Root system*

The sunflower's root system is of tap root type. The tap root may go down as far as five metres if conditions are favourable, but it has little real penetrating power. The sunflower also develops extensive superficial root hair growth. If conditions are favourable, root spread in young cultivated plants may amount to as much as 70 kg per hectare per day (Maertens and Bosc, 1981).

### *Above-ground vegetation*

The cultivated sunflower differs from the wild sunflower in that it has a single inflorescence (except male lines cultivated for seed production). The stem is topped by a single capitulum that may, in some cases, be very large.

Germination of the seed is epigeous. The height of the developed plant varies between 0.5 and 5 metres, but is usually 1.6 metres. The diameter of the plant's stem varies in the range of 0.5 cm to 10 cm. The size of a sunflower is related to the number of leaves and the duration of the "seed-to-flower" phase.

The stem has a tendency to bend slightly under the weight of the mature capitulum. The nature of this stem curve is largely under genetic control. The degree of stem curve is of fundamental importance since it determines the angle of the capitulum with respect to the stem and so the capacity to protect the florets and achenes from climatic stress (rain, hail, wind, sun) and birds (Bonjean, 1993; Seiler, 1997).

The leaf blade is continuous, cordate and irregularly toothed; it is frequently covered in short, hard hairs. It has pinnate veins, including three main veins. The first five pairs of leaves are opposite and the others are alternate, following a spiral phyllotaxy. The leaves may have a range of sizes and shapes, with the largest being between the fourth and tenth nodes. These are the intermediate leaves which play the most important part in the formation and accumulation of the seeds' fat reserves. It is worth noting that by the time the capitulum has formed the plant has developed almost half its total leaf surface, and by the beginning of flowering over 75% total leaf surface has developed (Merrien, 1986). The precise number of leaves may vary from 12 to 40, according to variety. However, the range is 20 to 40 in most of the hybrids currently cultivated (Bonjean, 1993).

### *The reproductive system*

The inflorescence is a capitulum the diameter of which may vary on average between 10 and 40 centimetres in most of the hybrids currently cultivated. The capitulum includes a fleshy receptacle which bears two types of flowers: ligulate flowers at the periphery, and tubular flowers in the centre. The edge of the capitulum is surrounded by leafy bracts arranged in overlapping concentric circles (2 to 5 in number).

The ligulate flowers form one or two rows around the periphery of the capitulum. There are never more than approximately thirty of these flowers. They are asexual, or, very rarely, unisexual of female type (Arnaud, 1986).

The tubular flowers or florets make up most of the capitulum. They are arranged in arcs which converge toward the centre of the capitulum. The florets are hermaphrodite and after pollination and fertilisation produce the achenes, which are harvested. The potential number of disk florets varies with capitulum diameter in the range of 60 to 3500.

### *Flowering*

The sunflower's flowering phase lasts between 9 and 15 days on average. The precise duration varies with the size of the capitulum and atmospheric conditions (Merrien, 1986). The flower unfolds centripetally from the periphery of the capitulum toward the centre (Marc and Palmer, 1978). Flowering begins with the ligulate flowers, which unfold their single petal immediately after the capitulum opens, and remain in flower until the florets have finished flowering (tubular flowers). The florets complete their flowering phase in 3–4 days in daily cycles involving one or two concentric rows. The flowering of each floret begins with the initial opening, its anthers projecting above the corolla with the extension of the filaments. The anthers are dehiscent and the pollen spills into the interior of the flower; this is the male stage. On the following day, the style extends through the interior volume of the flower and emerges above the anthers. The two stigmatic lobes separate and curl toward the style; this is the female stage. The stigma may remain receptive for 15 to 20 days (Arnaud, 1986; Bonjean, 1993).

### *Fertilisation*

The sunflower tends to be allogamous, using a complex system of sporophytic self-sterility. Nevertheless, the degree of self-incompatibility of the pollen varies widely and self-fertilisation remains a possibility (Bonjean, 1993), especially in the cultivated material.

The sunflower's pollen grains are relatively large (25  $\mu\text{m}$  to 35  $\mu\text{m}$ ). Each pollen grain comprises an outer coating (the exine) covered in sharp spines and a viscous wax. Due to these morphological characteristics, the pollen tends to form caked masses. As a consequence, it is usually carried by insects (Parker, 1981; Freund and Furgala, 1982; Bonjean and Pham-Delegue, 1986). An inventory carried out in France of pollinating fauna in a number of production areas has demonstrated that honey bees and bumble bees are the principal agents of pollination in the sunflower (Lecomte, 1962; Rollier, 1977; Madeuf and Leclercq, 1982). In other countries where sunflowers are cultivated, such as the United States or the Ukraine, surveys of pollinating insects reveal that domesticated bees are the principal pollinators (Bonjean, 1993).

It is unusual for the pollen to be carried on the wind; less than 0.2% of fertilisation occurs by wind pollination at a distance of less than one metre from the pollen source (Madeuf and Leclercq, 1982).

### *Fructification*

Sunflower seeds are achenes (or fruits) that consist of a kernel (true seed) and a pericarp (hull). The kernel consists of an embryo, endosperm, and seed coat. The pericarp (maternal tissue) consists of several layers: cuticle (external layer), epidermis, hypodermis, phytomelanin layer, fibrose tissue, and parenchymal layers adjacent to the kernel (Nassonov, 1940, quoted by Vranceanu, 1977). Pericarp colour is determined by the pigmentation of the epidermis, hypodermis, and phytomelanin layers (Putt, 1940; Mosjidis, 1982). The epidermis can be uniformly unpigmented or have black or dark brown stripes of varying width. The hypodermis is below the epidermis and can also be either completely unpigmented or pigmented (white or purple). The third layer (phytomelanin), if present, has uniform dark brown to black pigments (Putt, 1944).

The achenes located at the periphery of the capitulum are usually larger than those in the centre. While most florets form a shell, they may remain empty. This is because in many cases those in the central area do not produce seeds. As a consequence, there is a “sterile patch” in the centre of the capitulum the diameter of which depends not only on the genotype, but also on the conditions in which the sunflower is cultivated (Arnaud, 1986; Bonjean, 1993).

Sunflower seeds can be of two types:

- Oilseeds with an oil content greater than 40%, and 35% to 38% protein, usually black in colour.
- Edible seeds, which have a lower oil content (approximately 30%) and an outer shell that is usually dark brown or white.

### **3. The Physiological and Agronomical Characteristics of the Cultivated Sunflower**

#### **A. The sunflower growth cycle**

The complete growth cycle of the sunflower lasts between 100 and more than 170 days according to the variety and the growing conditions. Given this, and assuming zero growth below 6°C, the required accumulated temperature varies from 1500 °C to over 1700 °C. Flowering usually begins between 65 and 70 days after the emergence of the first shoots, which will occur once the accumulated temperature reaches 850°C, assuming a minimum growing temperature of 6°C (Merrien, 1986). Variations are found according to variety and location of cultivation, which suggests that there is a high degree of interaction with levels of illumination, to which the sunflower is very sensitive.

Sunflower is usually sown in the beginning of spring (February to May in the northern hemisphere) and harvested in late summer. The harvesting period extends from late August to September in the northern hemisphere, varying according to the region concerned.

The growth cycle can be divided into five phases (Rollier, 1972):

- The phase between sowing and the emergence of the first shoots

This phase lasts between 7 and 20 days. For emergence the temperature must be at least 4°C, the optimum level being around 8°C. This phase is important since it will determine the size of the plot's plant population.

- The phase between the emergence of the first shoots and growth of 4/5 pairs of leaves

This is the phase in which the root system is put in place and it is particularly sensitive to problems of soil structure due to errors in preparing for cultivation. While the rate at which dry matter is accumulated in the part of the plant above ground is high (10 kg per hectare per day), the dry matter in the roots represents approximately 15% of total dry matter in the plant. This is also the stage at which the initial formation of the leaves, and especially the flowers, begins. A lack of water at this point can limit leaf formation, but flower formation will be especially affected by low temperatures, which may lead to malformation of the capitulum.

- The phase between growth of the first five leaf pairs and the beginning of flowering

This is the most active growth phase for the crop, a phase during which the rate of formation of dry matter may be as high as 200 kg per hectare per day. The most spectacular increase is in leaf surface area, which is at a maximum during this period, as is also the case for the root system. This phase lasts between 40 and 50 days, and it is also the period of maximum intake of minerals such as nitrogen and boron.

- Flowering

The length of this phase varies slightly according to variety: 15 to 21 days for the plot as a whole, or 10 days for the individual plant. This is a period of limited growth during which the capitulum becomes the main sink for plant assimilates. During this phase, the sunflower is highly sensitive to low levels of moisture and infection of the capitulum by *Sclerotinia* spores.

- The seed-building phase

Levels of accumulated dry matter increase only very little in this phase, largely because during this period assimilates are reallocated and plant food reserves migrate. This is also the phase typified by active formation of fatty acids and new proteins from the amino acids derived from the breakdown of leaf and stem proteins. The total quantity of dry matter produced varies in the range of 10 to 15 metric tons per hectare (approximately 30 metric hundredweights per hectare). It may be as high as 20 tons per hectare for late hybrids grown in very good conditions. The point of physiological maturity is reached once the seeds have a moisture content of approximately 28%.

Nitrogen compounds, carbohydrates and fat contents vary during the sunflower growth cycle (Table 1.19).

**Table 1.19 Qualitative breakdown of plant dry matter (%) over the sunflower growth cycle.**

	Growth Stage								
	VE1 – Vegetative Emergence			F1 – Beginning of Flowering			M3 – Maturity		
	Nitrogen Compounds	Carbohydrates	Fats	Nitrogen Compounds	Carbohydrates	Fats	Nitrogen Compounds	Carbohydrates	Fats
Stem	15	15	-	13	37	-	6	25	-
Leaves	38	32	-	16	8	-	2	2	-
Capitulum	-	-	-	7	9	-	4	8	-
Seeds	-	-	-	-	-	-	11	7	35

Source : Merrien, 1986

The marketing norms for sunflower seeds require 9% moisture content and 2% impurities; at these levels, oil content marketing norm is 44%.

### B. The functioning of the plant and vegetation cover

The sunflower is characterised by a very high potential for photosynthesis compared with maize and soybean (Table 1.20). This performance can be explained by a number of factors:

- The numerous stomata distributed over the two leaf faces (surface and reverse side).
- Low resistance to diffusion of CO<sub>2</sub> from ambient air toward active photosynthesis sites.
- The very high level of RuBisCO activity, its high percentage in young leaves, and the greater accessibility of CO<sub>2</sub> to this enzyme in sunflowers compared with other crop plants.

**Table 1.20 Characteristics of the photosynthesis of selected crop plants**

Plant species	Carbon fixation mode	Enzymes involved	Average level of photosynthesis
<i>Helianthus annuus</i> (Sunflower)	C3	RuBisCO <sup>1</sup> + photorespiration losses	40 mg – 50 mg CO <sub>2</sub> /h/dm <sup>2</sup>
<i>Zea mays</i> (Maize)	C4	RuBisCO + PEPC <sup>2</sup> +PPDK <sup>3</sup> +NADP-ME <sup>4</sup> (little photorespiration)	40 mg CO <sub>2</sub> /h/dm <sup>2</sup>
<i>Glycine max</i> (Soybean)	C3	RuBisCO + photorespiration losses	20 mg CO <sub>2</sub> /h/dm <sup>2</sup>

<sup>1</sup> RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase; <sup>2</sup> PEPC: Phosphoenolpyruvate carboxylase; <sup>3</sup> PPDK : Pyruvate orthophosphate dikinase; <sup>4</sup> NADP-ME: NADP-Malic enzyme

Source: Merrien, 1986

The level of photosynthetic activity declines rapidly over time, largely as a result of shade and self-shading due to mutual coverage of vegetation. This decline is accelerated by dry conditions. In adult sunflowers, the best performance is found in the 15-20 leaf rank, which is the largest and therefore captures more illumination.

In the absence of limitations on the water regime, a sunflower will consume a great deal of water. It is capable of extracting large quantities from the soil if its root system is optimal.

Average daily water consumption may be up to 6 mm per day, with extreme daily values of 10 mm and above. This can be explained by the plant's high level of transpiration, at least double that of most species, which in turn is linked to the permeability of its leaves and the plant's overall high conductance.

When water is available in abundance, the sunflower tends to waste it. Conversely, in dry conditions, it is typically capable of regulating its consumption, improving the efficiency of water use. Thus the initial effect of limited water supplies will be reflected in the gradual closure of the stomata, leading to a reduction in water exchange, whereas photosynthesis will continue for some time.

There are two types of assimilate movement in the plant:

- Translocation, involving movements from the leaves (the location of biosynthetic processes) toward sink regions, where assimilates can accumulate (roots, stems, petioles, young growing leaves, capitulum).
- Tediistribution, involving movements of stored assimilates toward other plant organs (from the stem and leaves to the seeds, for example).

As soon as it is formed, the capitulum is the main sink site for assimilates. The biosynthesis of oil in the seeds occurs late and is essentially linked to the potential for post-flowering assimilation. It is preceded by protein synthesis, which uses amino acids previously held in store in the stems and leaves before being redistributed.

The quantity of protein present in the seed is largely dependent on the total quantity of nitrogen mobilised by the vegetation of the plant. Oil content is essentially related to the carbon fixation potential after flowering.

### C. Building sunflower yield

Sunflowers are grown in order to produce oil and seed cake. The plant's yield (in terms of oil or protein) can be broken down into a number of distinct components:

- The number of plants per hectare.
- The number of seeds per plant.
- The 1,000 kernel weight.
- The oil (and protein) content of the seeds.

Agronomic and plant physiology research directed at each of the above components of overall yield calls for a number of comments.

It is possible to modulate the "plants per hectare" parameter. This is because where plant density is high, the sunflower capitulum will be reduced in size; there will be more seeds, but each will be smaller. However, high densities increase lodging risks and facilitate the spread of plant diseases. It should also be borne in mind that it is preferable to ensure that the population is spread evenly over the plot, since sunflowers make poor use of free space.

The number of seeds per plant depends on the vigourousness of the plant concerned in its growth phase, total leaf area prior to flowering, and how long the foliage lasts after flowering. Capitulum vascularisation, a limiting factor in the central area, determines a quantitative and qualitative gradient for achene nutrition from the periphery toward the centre (see Table 1.21). A large-diameter capitulum can be seen to be an unsuitable objective in agronomic or genetic terms due to the limiting effect of vascularisation. Conversely, the search for varieties with an even distribution of vessels in the central area is a major goal for research and selection.

**Table 1.21 Characteristics of the seed in terms of its location on the capitulum**

Achene location	Average achene weight (mg)	Protein content		Oil content	
		(mg / achene)	(% of seed weight)	(mg / achene)	(% of seed weight)
Periphery	56.4	9.8	17.4	25.4	45.0
Median zone	50.5	9.9	19.6	19.7	39.0
Centre	44.8	9.8	21.8	16.0	35.7

Source : Merrien, 1986

The 1,000 kernel weight is largely dependent on how long the foliage lasts after flowering. It will vary with the position of the achenes on the capitulum, since those at the periphery are larger, although also less dense. The 1,000 kernel weight is always negatively correlated with the number of seeds. It compensates

only imperfectly and unpredictably for a reduction in the number of seeds. To conclude, average seed weight varies according to density (large achenes being associated with low density) as well as the variety concerned.

Yield varies widely according to the growing environment. Water is the main cause of such variation. There is no critical period in the cycle as is the case for maize; it is more the case that the sunflower is sensitive to lack of water throughout its growth cycle. This sensitivity is at its peak around the time of flowering. Water-related stress will affect mainly the number of achenes per plant; seed-filling (1,000 kernel weight) is less affected. Lastly, over-rapid senescence of the leaves following flowering will lead to a lower oil content.

Nitrogen plays a very important role in the phase in which the number of achenes is determined (differentiation), this being a major factor in the yield. However, despite the sunflower's high nitrogen requirement, it is usually fairly unresponsive to nitrogen-based fertilisation. Due to a low utilisation coefficient, nitrogen-based nutrition input to the plant generally takes the form of soil nitrogen.

#### 4 Possibilities of Crosses of Cultivated Sunflower with Wild Species

##### A. Intraspecific crosses

###### *Wild populations of H. annuus*

As has been mentioned in Section 1, the cultivated sunflower derives from a wild species (*Helianthus annuus*) originating in North America and domesticated in recent times. In the wild form, the plants are branched, producing large multiple heads that flower over long periods of time, bearing seeds that are small and present varying degrees of dormancy allowing them to remain in the soil for several years.

In the United States, such wild populations are present in the sunflower cultivation area and genes may be easily exchanged between wild and domesticated populations through cross-pollination. The frequency of hybridisation is unknown, but the phenomenon is a recurrent one even when the wild species are several kilometres away from the sunflower fields (Faure *et al.*, 2002).

In Europe, several sub-spontaneous populations of wild *H. annuus* were observed, which are now increasing especially in some places in central Italy and Andalusia, Spain (Faure *et al.*, 2002). The origin of these invading populations in Europe is under question (for example, through wild seed importation or de-domestication).

Due to the fact that such exchanges are possible, and in order to maintain purity of commercial and basic seeds, fields used for sunflower seed production in the USA are kept at least 800 metres distance from commercial sunflower fields and wild sunflower populations. For basic seed production, this distance, which was set initially at 3 km, has been increased to 6.4 km from commercial sunflower fields, 3.2 km from seed production locations and 4.8 km from oilseed crops. In Europe, the production of sunflower seeds requires at least 500 metres' separation from all other commercial sunflower crops, and 3 km to 5 km in the case of basic seed production (Faure *et al.*, 2002).

Wild populations of *H. annuus* are also present in Mexico, Canada, Australia and Argentina.

From extensive intraspecific crossing experiments, Heiser (1954) indicates that the *H. annuus* species are cytologically uniform and that intraspecific crossability level is high. In natural conditions, Rieseborg *et al.* (1998) showed that hybrids between cultivated and wild *H. annuus* occurred frequently. As much as 42% of progenies from wild plants near cultivars were hybrids, and cultivar genes have been shown to

persist in wild populations for several generations. The conclusion was that introgression of cultivar loci is widespread in the sympatric wild *H. annuus* populations (Linder *et al.*, 1998).

#### *Volunteer populations*

Sunflower seeds may stay in the soil after harvesting and germinate several years later, thus creating quasi-self-sown or volunteer populations along the edges of fields and within later crops in the rotation (fallow, peas, soybean, maize, sunflower).

Unlike the wild populations of *H. annuus*, which have been thoroughly studied in the United States, little information, either in agronomic or genetic terms, is available on this topic in Europe. However, European wild populations of sunflower derive from North America seed import. Therefore, the knowledge of sunflower wild population biology and of management practices accumulated in the United States provides valuable information for Europe as well.

There have been no indications that such self-sown growth is problematic or adventive in relation to crops either in the United States or in Europe. It is usually eliminated over the two years following the harvesting of the sunflower crop (Snow, 1999).

### **B. Related *Helianthus* species and interspecific hybridisation**

In Europe, three wild annual species related to sunflower have been observed (*H. bolanderi*, *H. argophyllus* and *H. debilis*). They tend to be found more in private gardens than in sunflower growing areas.

Considering the annual wild species of the section *Helianthus* (*H. argophyllus*, *H. petiolaris*, *H. debilis*, *H. praecox*, *H. bolanderi*, *H. niveus*, *H. neglectus*, *H. paradoxus*; *H. anomalus*, *H. deserticola*), interspecific hybrids may be obtained (more or less easily) in crossings with the cultivated sunflower *H. annuus*, with (or without) embryo rescue techniques (Whelan, 1978; Christov, 1996). In such interspecific hybrids, semi-sterility is a common trait due to strong genetic barriers: chromosomal translocations, inversions, etc. (Whelan, 1978; Heiser *et al.*, 1969). Viable hybrids and fertile interspecific progenies are generally produced. In natural conditions, hybrid zones are frequently observed in the United States, and various experimental procedures have revealed such gene flows between *H. annuus* and other wild species *H. argophyllus*, *H. bolanderi*, *H. debilis* and *H. petiolaris* (Rieseberg *et al.*, 1998).

Among the perennial species related to the sunflower which are present at a significant level in Europe, there are two hexaploid forms belonging to the *Atrorubens* section, one in the *Corona-solis* series (*H. tuberosus*) and one in the *Atrorubentes* series (*H. rigidus*).

*H. tuberosus* (Jerusalem artichoke) is a species still grown for its tubers and is used in animal feed. It is found in many places in France, Montenegro and Yugoslavia, as well as in central and eastern Europe.

Today, there are numerous self-sown populations of Jerusalem artichoke in a range of geographical regions but there is little information on their distribution or frequency.

*H. rigidus* is also a decorative perennial form frequently found in private gardens.

In Europe, the common forms of *H. tuberosus* and *H. rigidus* flower in September and in theory there is no overlap with the flowering stage of large-scale sunflower crops, at least where these are sown in spring. However, in botanical collections wild ecotypes exist that flower early, similarly to cultivated sunflowers.

Artificial F1 hybrids between the cultivated sunflower *H. annuus* L. and many perennial species of the *Atrorubens* section may be obtained, but they are difficult to perform due to the strong genomic and chromosomal divergencies. Successful hybridisation results are reported, with variable sterility levels of their F1 hybrids, for the following perennial species: *H. angustifolius*, *H. californicus*, *H. decapetalus*, *H. divaricatus*, *H. eggertii*, *H. floridanus*, *H. giganteus*, *H. glaucophyllus*, *H. grosseserratus*, *H. hirsutus*, *H. laevigatus*, *H. maximiliani*, *H. microcephalus*, *H. mollis*, *H. nuttallii*, *H. occidentalis*, *H. resinosus*, *H. rigidus*, *H. salicifolius*, *H. smithii*, *H. strumosus*, *H. tuberosus* (Whelan, 1978; Christov, 1996; Gravilova *et al.*, 2000).

In artificial conditions (isolation cages with bees), the hybridisation level of cultivated sunflower with *H. tuberosus* is low and F1 seed set varies in the range of 2-5%, according to accessions (H. Seriesy, Pers. comm.).

Partial hybridisation between perennials and sunflower was observed under artificial crossing conditions (Faure *et al.*, 2002). The phenotype and genotype of F1 hybrids was very close to the female parent. This phenomenon, if observed in natural conditions, could be an opportunity for gene-flow from cultivated sunflower to the wild perennial forms.

In natural conditions, interspecific crosses within *Atrorubens* section species frequently occurred (Heiser *et al.*, 1969), but little information is available on the natural crossings between sunflower and perennial species. Crosses of sunflower with the species of the *Ciliare* section appeared rather uncommon in natural conditions and the rare hybrids obtained via embryo rescue techniques exhibited strong sterility.

## 5 Potential Interactions with Other Organisms

### A. Sunflower insects

Several insect species attack sunflower (*Helianthus annuus* L.) worldwide. In North America, a large pest complex has evolved on wild sunflower and has moved from wild ancestors to commercial cultivars. In other countries and to a lesser extent in North America, some insects have adapted to utilise sunflower as an alternative host. Many of these insects develop or increase in number on earlier-planted crops and then after senescence move to sunflower.

The table in Appendix 1 is intended as an identification guide for categories of insects which interact with the cultivated and stored *H. annuus*. This table has been established from the article of Charlet *et al.* (1997) and summarises the present state of knowledge on this subject. It is representative of every continent, but should be probably completed by each environmental safety assessor.

### B. Sunflower diseases

The distribution of sunflower pathogens around the world has followed the introduction of sunflower into each continent. So, most pathogens of sunflower can be found in every country today. However, with differences in climate and cultural practices among countries, the prevalence and the incidence of specific diseases will vary in each country.

The sunflower diseases related to the causal organisms are presented in Appendix 2.

### **C. Other sunflower consumers**

#### *Rabbits and hares*

Some damage can be caused by rabbits and hares at the early sunflower stages, particularly when the fields are planted near woods or set-aside lands. They damage sunflower by cutting the stem of plant from 2 to 5 mm above the soil surface or by eating young leaves. Important losses have already been observed.

#### *Birds*

Bird damage is a problem in every sunflower-growing region of the world. It occurs from early maturation to harvest but seems greatest within 18 days after anthesis. Small sparrows (*Passeridae*) to large species such as crows (*Corvidae*) and parrots (*Psittacidae*) eat sunflower achenes (easily obtained) or seeds (Linz and Hanzel, 1997). The losses can be economically severe.

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## APPENDIX I

## Insect Pests in Sunflower

Insect pests	CONTINENT OR REGION IN WHICH THEY OCCUR						
	USA/CANADA /MEXICO	SOUTH AMERICA	AFRICA	WESTERN EUROPE	CENTRAL & EASTERN EUROPE	ASIA	AUSTRALIA
<b>Cutworms [Lepidoptera : Noctuidae]</b>							
Dark-sided cutworm <i>Euxoa messoria</i> (Harris), <i>Euxoa tenax</i> (Hübner)	X				X X		
Red-backed cutworm <i>E. ochrogaster</i> (Guenee)	X						
Dusky cutworm <i>Feltia diocens</i> (Walker)	X						
<b>Sunflower Bud Moth,</b> <i>Satonia helianthana</i> (Riley) [Lepidoptera : Tortricidae]	X (Mexico to the central USA)						
<b>Sunflower Stem Weevil,</b> <i>Cyindrocopturus aspersus</i> (LeConte) [Coleoptera : Curculionidae]	X						
<b>Black Sunflower Stem Weevil,</b> <i>Aptus occidentalis</i> Fall [Coleoptera: Curculionidae]	X (North Dakota, Minnesota & Texas)						
<b>Sunflower Root Weevil,</b> <i>Borus strepera</i> (LeConte) [Coleoptera : Curculionidae]	X (Illinois to California & Montana to Guatemala)						
<b>Sunflower Maggot,</b> <i>Stratiotia longipennis</i> (Wiedemann) [Diptera : Tephritidae]	X (USA, Canada)						
<b>Long-horned Sunflower Stem Girdler,</b> <i>Dectes texanus</i> LeConte [Coleoptera: Cerambycidae]	X (North & South Dakota, Florida)						
<i>Ligyrus gibbosus</i> (DeGree) [Coleoptera: Scarabaeidae]	X (USA, southern Canada, & northern Mexico)	X					
<i>Ligyrus</i> spp. (Scarabaeidae).		X					

Insect pests	CONTINENT OR REGION IN WHICH THEY OCCUR						
	USA/CANADA /MEXICO	SOUTH AMERICA	AFRICA	WESTERN EUROPE	CENTRAL & EASTERN EUROPE	ASIA	AUSTRALIA
<b>Grasshoppers [Orthoptera: Acrididae]</b> <b>The most important :</b> <i>Melanoplus differentialis</i> (Thomas) ; Migratory grasshopper, <i>M. sanguinipes</i> (Fabricius) ; Twostriped grasshopper, <i>M. bivittatus</i> (Say) , Redlegged grasshopper, <i>M. femurrubrum</i> (DeGeer) Clearwinged grasshopper, <i>Canisula pellucida</i> (Scudder) <i>Dichroplus platensis</i> (Burner) ; <i>D. conspersus</i> (Burner).	X X X X X	X X					
<b>Painted Lady or Thistle Caterpillar,</b> <i>Vanessa cardui</i> (L.) [Lepidoptera: Nymphalidae]	X						
<b>Sunflower Beetle,</b> <i>Zygogramma exclamationis</i> (Fabricius) [Coleoptera: Chrysomelidae]	X						
<b>Sunflower Moth,</b> <i>Homocidus electellus</i> (Hbst) [Lepidoptera: Pyralidae] <i>Homocidus nebulosus</i> Denis and Schiffermüller <i>Homocidus heliophili</i> (Pastrana) <i>Homocidus viciarius</i> (Pastrana),	X	X (Argentina) X (Argentina)		X X	X	X (Iran)	
<b>Sunflower Midge,</b> <i>Contarinia schulzi</i> (Gagne) [Diptera: Cecidomyiidae]	X						
<b>Sunflower Seed Midge</b> <i>Neodaxiptera helianthi</i> (Felt) [Diptera: Cecidomyiidae]	X						
<b>Red Sunflower Seed Weevil,</b> <i>Sitona fuscus</i> (LeConte) [Coleoptera: Curculionidae]	X						
<b>Gray Sunflower Seed Weevil,</b> <i>Sitona sordidus</i> (LeConte) [Coleoptera: Curculionidae]	X						
<b>Banded Sunflower Moth,</b> <i>Cochylis hospes</i> (Walsingham) [Lepidoptera: Cochylidae]	X (USA, Canada)						
<b>Sunflower Receptacle Maggot,</b> <i>Gymnocyrtus diffusa</i> (Snow) [Diptera: Tephritidae]	X Great plains from Montana south to Arizona, east to Missouri						

Insect pests	CONTINENT OR REGION IN WHICH THEY OCCUR						
	USA/CANADA /MEXICO	SOUTH AMERICA	AFRICA	WESTERN EUROPE	CENTRAL & EASTERN EUROPE	ASIA	AUSTRALIA
<b>Sunflower Seed Maggot,</b> <i>Neotephritis flalis</i> (Loew) [Diptera : Tephritidae]	X (Southern Canada to northern Mexico; throughout continental North America)						
<b>Sunflower Headclipping Weevil,</b> <i>Haplorhynchites aeneus</i> (Boheman) [Coleoptera : Curculionidae]	X (USA, Canada)						
<b>Nymphalid butterfly,</b> <i>Chloryne lacinia saundersii</i>		X (Brazil)					
<b>Noctuid,</b> <i>Rachiphius na</i> (Guenee)		X (Argentina)					
<b>The black cutworm,</b> <i>Agrotis ipsilon</i> (Hufnagel) <i>Agrotis zageana</i> (Denis, Schiff.) [Noctuidae] <i>Agrotis</i> spp. [Lepidoptera: Noctuidae] brown cutworm, <i>Agrotis mundana</i> (Walker); bogong moth <i>A. infusa</i> (Boisduval); variable cutworm, <i>A. prophyrcollus</i> (Guenee). Pale western cutworm <i>Agrotis orthogonia</i> (Morrison)	X	X (Brazil)	X X	X		X (Iran)	X X X X
<b>The chrysomelid,</b> <i>Diabrotica speciosa</i> (Germar)		X					
<b>The scarab beetle,</b> <i>Cyclocephala melanocephala</i> (Fabricius)		X (Brazil)					
<b>Aphids</b> [Homoptera: Aphididae], <i>Bemisia</i> sp. ;		X					
<b>The Brazilian leafhopper,</b> <i>Protaetia brella brasiliensis</i> (Baker) ;		X					
<i>Empoasca</i> sp. [Cicadellidae] The leafhoppers, <i>Empoasca pteridis</i> Dhlb. ; <i>Empoasca devastans</i> (Davi.)		X		X			
<i>Liriomyza</i> sp. (Agromyzidae) ; <i>Leptocortica tipuloides</i> (DeGeer) (Coreidae) ;		X X			X		X
<b>The noctuids,</b> <i>Pseudoplata includens</i> (Walker),		X				X	
<b>The velvetbean caterpillar,</b> <i>Anticarsia gemmatilis</i> ( Hubner),		X					
<b>The fall armyworm,</b> <i>Spodoptera frugiperda</i> (Smith) <i>Spodoptera exigua</i> (Hubner)		X					

Insect pests	CONTINENT OR REGION IN WHICH THEY OCCUR						
	USA/CANADA /MEXICO	SOUTH AMERICA	AFRICA	WESTERN EUROPE	CENTRAL & EASTERN EUROPE	ASIA	AUSTRALIA
<i>Spodoptera litura</i> (Fabricius)			X (Egypt)		X		
<b>Caterpillar [Nymphalidae],</b> <i>Actinote pellena pellena</i> (Hubner)		X (Argentina, Venezuela, Brazil, Paraguay, Uruguay Peru)					
<b>The yellow woolly-bear,</b> <i>Spilosoma virginica</i> (Fabricius) <i>Spilosoma carignatum</i> (Kollar) <i>Spilosoma obliqua</i> (Walker)		X (Argentina)				X X	
<b>The spotted maize beetle,</b> <i>Arytus aromaculatus</i> Blanchard (Melyridae)		X (Argentina)					
<i>Athamastus haematodes</i> (Stal) [Coreidae];		X					
<i>Edessa medialis</i> (Fabricius) [Pentatomidae]		X (Argentina)					
<i>Nezara viridula</i> (L.) [Pentatomidae]		X	X (Egypt)				X
<i>Gargaphia torresii</i> (C.L.) [Tingidae]		X					
<b>the black cutworms ;</b>		X					
<b>the variegated cutworm,</b>		X					
<i>Pendroma saucia</i> (Hubner) (Noctuidae);		X					
<i>Melanogramyza cunctatorides</i> (Blanchard) [Agromyzidae];		X					
<i>Hylemyia</i> spp. [Anthomyiidae];		X					
<i>Disonychodes exclamatoria</i> (Bohemian) (Chrysomelidae);		X					
<i>Conoderus</i> spp. (Elateridae);		X					
<i>Epicauta leopoldina</i> (Haag-R.) (Meloidae)		X					
<i>Acromyrmex</i> spp. (Formicidae);		X					
<i>Acromyrmex heyeri</i> (Forcl)		X (Uruguay)					
<i>Difoboderus abderus</i> (Sturm.)		X					
<i>Discynetus gages</i> (Burmeister)		X					
<i>A. lindi</i> (Guerin)		X (Uruguay)					
<i>B. striatus</i> (Roger)		X					
<b>The Scarabs of the genera,</b>							
<i>Phyllophaga</i> , <i>Dyscinetus</i> , <i>Phileurus</i>		X					
<b>Larvae of <i>Hylemyia ciliatula</i> (Rondani) [Anthomyiidae]</b>		X					
<i>Myzus persicae</i> (Sulzer)		X (Uruguay)				X	
<i>Mallocephala deserticola</i> (Bergman) [Noctuidae]		X					
<i>Schizomycha</i> sp. [Scarabaeidae]			X (Nigeria)				
<i>Gonocephalum simplex</i> (Fabricius) [Tenebrionidae]			X				

Insect pests	CONTINENT OR REGION IN WHICH THEY OCCUR						
	USA/CANADA /MEXICO	SOUTH AMERICA	AFRICA	WESTERN EUROPE	CENTRAL & EASTERN EUROPE	ASIA	AUSTRALIA
<b>The noctuid,</b>							
<i>Plutia orchalica</i> (Fabricius)			X			X	
<i>Plutia</i> spp. (Noctuidae)						X	
Piercing-sucking Hemiptera			X (Nigeria)				
<i>Macrostelus</i> sp. [Cicadellidae]			X				
<i>Dacus curcurbitae</i> Coquillett [Tephritidae]			X				
<b>The noctuid moth, [Lepidoptera: Noctuidae]</b>							
<i>Helicoverpa armigera</i> (Hübner)			X		X	X	X
<i>Helicoverpa</i> spp.							X
<i>Helicoverpa punctigera</i> (Wallengrén)							X
<i>Colletes dreyer</i> (Germar)			X				
<i>C. boheman</i> (Stål)			X				
<i>Nysius stali</i> (Evans) [Lygaeidae]			X				
<i>Lygus</i> spp. (Miridae)				X			
<i>Lygus pratensis</i> L. (Hemiptera: Miridae)				X			
<i>Lygus rugulipennis</i> (Poppo) [Miridae]				X	X		
<i>Lygus Gemelatus</i> (HS)					X		
<b>The aphids,</b>				X (Germany)	X (Yugoslavia, Hungary, Romania, Bulgaria, former USSR)		
<i>Brachycaudus helichrysi</i> (Kaltenbach)							
<i>Aphrodes brevincher</i> (Scheink) [Homoptera: Cicadellidae]				X			
<b>Aphids [Homoptera: Aphididae]</b>							
<i>Aphis fabae</i> Scop.				X	X	X	
<i>Aphis gossypii</i> (Glover)							
<i>Aphis helianthi</i> (Monell)	X						
<i>Masonaphis masoni</i> (Knowlton)	X						
<i>Dolycoris baccarum</i> (L.) (Hemiptera: Pentatomidae);							
<i>Ostrinia sublaeta</i> (Hübner) ,				X			
<i>Phytocnoides sticticollis</i> (L.) (Lepidoptera: Pyralidae);				X			
<i>Phytomyza geniculata</i> Macq. (Diptera: Agromyzidae).				X			
<i>Acanthophilus helianthi</i> (Rossi) [Diptera: Tephritidae]				X (Italy)			
<i>Eurydema ventrale</i> Kolenat [Hemiptera: Pentatomidae]					X (Italy)		
<i>Opastion sublaetum</i> L. (Coleoptera: Tenebrionidae)				X (France)			
<i>Eupteryx atropunctata</i> (Goeze) (Cicadellidae); The				X			
leafminer, <i>Phytomyza horticola</i> (Gowatou)				X			

Insect pests	CONTINENT OR REGION IN WHICH THEY OCCUR						
	USA/CANADA /MEXICO	SOUTH AMERICA	AFRICA	WESTERN EUROPE	CENTRAL & EASTERN EUROPE	ASIA	AUSTRALIA
<i>Acheta deserti</i> Pall. [Orthoptera: Gryllidae]					X (Yugoslavia Hungary Romania and the former USSR)		
<i>Lehrus apterus</i> (Laxm.) [Coleoptera: Scarabaeidae]					X (Bulgaria, former USSR)		
<b>The Wireworms</b> [Coleoptera: Elateridae] <i>Agriotes</i> (especially <i>A. ustulatus</i> Schall. <i>A. sputator</i> L., <i>A. gurgistanus</i> Fald, <i>A. ponticus</i> Stepanov, <i>A.</i> <i>lineatus</i> L., <i>A. obscurus</i> L.). <i>Sclatoronus</i> and <i>Melanotus</i> (including <i>M. fusciceps</i> Gyll.) <i>Athys haemorrhoidalis</i> (Fabricius)					X X X X X X		
<i>Psidium macillosum</i> (Fabricius) [Coleoptera: Curculionidae]					X (Yugoslavia, Hungary Bulgaria, former USSR)		
<i>Tanyecus difanollis</i> Gyllenhal [Coleoptera: Curculionidae]					X (Yugoslavia, Hungary Bulgaria, former USSR)		
Miscellaneous Coleoptera [Cerambycidae, Mordellidae] <i>Agapanthus dahl</i> (Richt)					X		
<b>Beet Webworm,</b> <i>Loxostege sticticalis</i> (L.) [Lepidoptera: Pyralidae]					X (Yugoslavia, Hungary Romania, Bulgaria, former, USSR)		
<i>Mamestra brassicae</i> L. [Lepidoptera: Noctuidae]					X		
<i>Adelphocoris lineolatus</i> (Goeze),	X (North America)						
Miscellaneous Plant Bugs [Pentatomidae, Lygaeidae, Coreidae]					X		
<b>Tingidae</b> (Hemiptera), <i>Galeatus belamhi</i> (Onder and Lodos) <i>Galeatus scrophiensis</i> (Saunders)						X (Turkey)	
<i>S. littoralis</i> (Boisduval) (defoliation),						X	
<b>Strawberry spider mite,</b>						X (Iran)	
<i>Petranychus naristani</i> (Ugarcin and Nikolski)						X (Iran)	
<b>The western flower thrips,</b> <i>Frankliniella occidentalis</i> (Pergande) <b>Thrips</b> [Thysanoptera],						X (Israel)	

Insect pests	CONTINENT OR REGION IN WHICH THEY OCCUR						
	USA/CANADA /MEXICO	SOUTH AMERICA	AFRICA	WESTERN EUROPE	CENTRAL & EASTERN EUROPE	ASIA	AUSTRALIA
<i>Thrips tabaci</i> (Linderman), <i>Frankliniella schultzei</i> (Trybom), <i>Desmoultriea tenuicornis</i> (Bagnall), <b>the plague thrips, <i>T. iswaginis</i> (Bagnall)</b>							X X X X
Predators: <i>Orius</i> spp.						X	
<i>Osmia damosa</i> Walker (Lepidoptera: Pyralidae)						X	
<i>Rhopalosiphum erysimi</i> (Kaltenbach)						X	
<i>Carpis unipuncta</i> (Haw.)						X	
<i>Carpis loreyi</i> (Dupt.)						X	
Grasshoppers, <i>Carpophylus</i> sp. (Nindulidae) ; <i>Disomcha</i> sp. (Chrysomelidae) ;						X X	
Leafhopper (Homoptera: Cicadellidae), <i>Amrasca biguttula</i> <i>biguttula</i> (Ishida)						X	
<i>Phytomyza atricornis</i> (Meigen) (Diptera: Agromyzidae)						X	
Black Scarab Beetles, <i>Pseudoheteronyx</i> spp. (Coleoptera: Scarabaeidae)							X
False Wireworms [Coleoptera: Tenebrionidae] <b>the striate false wireworm,</b> <i>Pterohelaeus alternatus</i> Pascoe, <b>the eastern false wireworm,</b> <i>P. darlingensis</i> Carter ; <b>the southern false wireworm,</b> <i>Gonocephalum macleayi</i> (Blackburn) <b>Another species,</b> <i>Celibe</i> sp. (= <i>Saragus</i> sp.)							X (South Australia, New South Wales)  X  X  X
Wingless Cockroaches, <i>Calolampyris</i> spp. (Orthoptera: Blaberidae) <i>Calolampyris elegans</i> Roth and Princis and <i>C. solida</i> Roth and Princis							X (Central Highlands of Queensland)
<b>Black Field Earwig, <i>Naisa fivdipes</i> (Dufour) (Dermaptera: Labiduridae)</b>							X
<b>Field Crickets,</b> <i>Teleogryllus</i> and <i>Lepidogryllus</i> spp. (Orthoptera: Gryllidae)							X
<b>Black field crickets</b> <i>Teleogryllus commodus</i> (Walker) and <i>T. oceanicus</i> (Le Guillou), <b>Brown field crickets,</b> <i>Lepidogryllus parvulus</i> (Walker) and <i>L. comparatus</i> (Walker).							X  X

Insect pests	CONTINENT OR REGION IN WHICH THEY OCCUR						
	USA/CANADA /MEXICO	SOUTH AMERICA	AFRICA	WESTERN EUROPE	CENTRAL & EASTERN EUROPE	ASIA	AUSTRALIA
<b>Sugarcane Wireworm</b> <i>Agrypnus variabilis</i> (Candèze) [Coleoptera: Elateridae]							X
<b>Soybean Looper,</b> <i>Thysanophora orichalcea</i> (Fabricius) [Lepidoptera: Noctuidae]							X
<b>Greenhouse Whitefly,</b> <i>Trialeurodes vaporariorum</i> (Westwood) [Hemiptera: Aleyrodidae]							X
<b>Rutherglen Bug,</b> <i>Nysius vinitor</i> (Bergroth) [Hemiptera: Lygaeidae] <i>N. clevelandensis</i> (Evans)							X X (Queensland and northern New South Wales)

**Insect pests of stored sunflower**

the sawtoothed grain beetle ( <i>Oryzaephilus surinamensis</i> L.) (Coleoptera: Cucujidae),
red flour beetle ( <i>Tribolium castaneum</i> (Herbst)) (Coleoptera: Tenebrionidae),
Indian meal moth ( <i>Plodia interpunctella</i> (Hübner)) (Lepidoptera: Pyralidae)

## APPENDIX II

The Pathogens of Sunflower (Classification proposed by Gulya *et al.*, 1997)

Disease	Causal organism	Country or region in which they occur
Downy mildew	<i>Plasmopara halstedii</i> (Farl.) Oomycetes	Every continent with the exception of Australia.
Sunflower rust	<i>Puccinia helianthi</i> (Schwein.) <i>Puccinia xanthii</i> (Schwein.)	Worldwide. Only in Australia.
Alternaria	<i>Alternaria helianthi</i> (Hansf.), syn. <i>Helminthosporium helianthi</i> Hansf.) <i>Alternaria zinnia</i> (Pape) <i>A. helianthinificiens</i> (Simmons)  <i>A. helianthicola</i> (Rao & Rajagopalan) <i>A. protenta</i> (Simmons) <i>A. tenuis</i> Nees (Simmons)	Worldwide.  Worldwide. North Dakota, Manitoba, Hungary; Yugoslavia. India, Yugoslavia. Uganda, Rhodesia. India, Iran.
Septoria leaf spot Septoria leaf speck	<i>Septoria helianthi</i> (Ell & Kell) <i>Septoria helianthina</i> (Petrov & arsinjevic)	Worldwide except South America. Yugoslavia.
Bacterial foliar diseases	<i>Pseudomonas tagetis</i> , reclassified as <i>Pseudomonas syringae</i> (Ps) pv. <i>tagetis</i> (Hellmers) Young, Dye, Wilkie ; <i>Pseudomonas syringae</i> pv. <i>helianthi</i> (Kawanua) Young, Dye, Wilkie. <i>Pseudomonas cichorii</i> (Swingle) <i>Xanthomonas campestris</i> pv. <i>phaseoli</i>	Every continent.  Japan, Canada, Mexico, USA, Europe, Africa, India, New Zealand. Subtropical climates, Brazil. India and Russia.
Powdery mildews	<i>Erysiphe cichoracearum</i> DC. ex. Meret  <i>Sphaerotheca fuliginea</i> (Schlecht. ex Fr.) Poll <i>Leveillula tarucia</i> (Lev.) Arn.	All continents.  Africa, Asia, Europe, and South America. China, India, the former Soviet republics, and the Middle East.
White Rust	<i>Albugo tragopogonis</i> (DC.) S. F. Gray [Syn. = <i>Albugo tragopogi</i> (Pers) Schroet]	Every continent
Virus diseases		
Aster Yellows (rarely observed)	Mycroplamas	USA, Canada, Argentina.
	Other sunflowers diseases with mycoplasmas	France, Israël, India, Sudan.
Cucumber Mosaic	Cucumber Mosaic Virus (CMV)	China, India Once from a nursery in Maryland.
Sunflower Mosaic	Sunflower Mosaic Virus (Potyvirus) (SMV)	Argentina, Texas, Czech Republic.
Sunflower Ringspot	Sunflower Ringspot Virus (Ilarvirus) (SRV)	Queensland, Australia.
Sunflower Yellow Blotch and Leaf Crinkle	Luteovirus	African countries and England.
Tobacco Ringspot	Tobacco Ringspot Virus (Nepovirus) (TRV)	Rio Grande Valley of Texas (on wild <i>H. annuus</i> ).
Tobacco Streak	Tobacco Streak Virus (Lilarvirus) (TSV)	On garden sunflower in the Netherlands.
Tomato Spotted Wilt	Tomato Spotted Wilt Virus (Tospovirus) (TSWV)	Ukraine.
Minor Foliar Diseases (leaf spots)	<i>Ascochyta compositarum</i> (J.J. Davis) – Coelomycetes	USA, Kenya, Japan, Russia.

	<i>Cercospora helianthi</i> (Ell & Ever) – Hyphomycetes ; <i>Cercospora helianthicola</i> (Chupp & Viegas) <i>Cercospora pachyrus</i> (Ell & Kellerman)	USA, Brazil, Russia.
	<i>Colletotrichum helianthi</i> J.J. Davis - Coelomycete	-
	<i>Entyloma compositarum</i> Farl - Tilletiaceae	Montana.
	<i>Epicothium neglectum</i> Desm. - Hyphomycetes	Yugoslavia, Romania.
	<i>Itersonilia perplexans</i> Derx - Basidiomycete	Canada, Uruguay.
	<i>Myrothecium roridum</i> Tode:Fr. (Alb. & Schw.) – Hyphomycetes	Pakistan.
	<i>M. verrucaria</i> Ditmar:Fr – Hyphomycetes	Argentina.
	<i>Phialophora asteris</i> (Dowson) Burge & Isaac f. sp. <i>Helianti</i> Tirilly & Moreau – (Soilborne fungus)	Canada, Italy.
	<i>Phyllosticta Wisconsinensis</i> H.C. Green - Coelomycete	-
	<i>Sordaria fimicola</i> (Rob. Ex Desm.) Ces & Not. - Ascomycete	Yugoslavia, USA.
Miscellaneous Foliar Pathogens		
	Species of : <i>Botryodiplodia</i> , <i>Cladosporium</i> , <i>Corynespora</i> , <i>Curvularia</i> , <i>Helminthosporium</i> , <i>Mycosphaerella</i> , <i>Pyrenophora</i> , <i>Sphaceloma</i>	Tropical climates.
Sclerotinia Wilt	<i>Sclerotinia sclerotiorum</i> (Lib.) de Bary	Worldwide.
	<i>Sclerotinia minor</i> Jagger	Australia, Argentina, Uruguay, Chile, California.
Phomopsis Stem Canker	<i>Phomopsis helianthi</i> Munt.-Cvet <i>et al.</i>	Worldwide
Phoma Black Stem	<i>Phoma macdonaldii</i> (Boerema)	Northern Great Plains of USA, California, Kansas. Countries of Africa, Asia (with the exception of China), Argentina, Europe.
Verticillium Wilt/Leaf Mottle	<i>Verticillium dahliae</i> (Klebahn)	Europe, Argentina, Mexico, former USSR, England, north-central plain in the USA, Canada.
Charcoal Rot	<i>Macrophomina phaseolina</i> (Tassi) Goid Synonyms <i>Sclerotium bataticola</i> Tabu and <i>Rhizoctonia bataticola</i> (Taub.) Briton Jones.	Most around the world but more prevalent in Egypt, India and Pakistan.
Southern blight or collar rot	<i>Sclerotium rolfsii</i> Sacc. (syn. <i>Corticium rolfsii</i> Curzi) - Basidiomycete	Tropical and subtropical climates.
Minor root and stalk pathogens :		
Stem rot	<i>Phytophthora cryptogea</i> Pethyb. & Kaff.	California, Iran.
Root rots and seedling damping off	Several species of <i>Pythium</i> including <i>P. aphanidermatum</i> (Edson) Fitzp., <i>P. debaryanum</i> Auct. Non-Hesse, <i>P. irregulare</i> Buisman, <i>P. rostratum</i> Butler <i>P. splendens</i> Braun	USA, Iran.
Texas root rot	<i>Phymatotrichum omnivorum</i>	Texas.
Wilt	<i>Fusarium moniliforme</i> (sheld) <i>Fusarium oxysporum</i> (Schlect) <i>Fusarium tabacinum</i> (Beyma)	India, North America. India, North America. Italy.
Sclerotinia Head rot	<i>Sclerotinia sclerotiorum</i>	Argentina, several European countries, Japan, North America.

Rhizopus head rot	<i>Rhizopus</i> (at least three species)	Australia, India, South Africa, USA, Canada, the Mediterranean areas of Europe, Egypt and Russia
Botrytis head rot	<i>Botrytis cinerea</i> Pers.	All European countries, Egypt, Turkey, India, Pakistan, Russia, Canada, USA
Bacterial head rot	<i>Erwinia carotovora</i> Jones, Holland	USA, Mexico, several European countries, several central African countries, Russia
Nematodes	<i>Meloidogyne</i> spp. (Volvas & Sassanelli)  <i>Meloidogyne incognita</i> <i>Meloidogyne javanica</i> <i>Rotylenchulus</i> (Robinson & Orr) <i>Tylenchorhynchus</i> (stunt), <i>Helicotylenchus</i> (spiral),  <i>Pratylenchus</i> (pin),  <i>Xiphinema</i> (dagger), <i>Hoplolaimus</i> (lance), <i>Quinisulcius</i> (stunt) <i>Trichodorus</i> <i>Belonolaimus</i> <i>Scutellonema</i> , <i>Paratrichodorus</i> , <i>Rotylenchus</i>	California, Florida, Tennessee, Texas, India, South Africa, Italy, Egypt, Serbia. Brazil, Egypt, India, Italy, Serbia, South Africa, Zambia. Brazil, Egypt, India, Italy, Serbia, South Africa, Zambia. India. California. California, Florida, Tennessee, Texas. California, Florida, Tennessee, Texas. California.  Florida, Tennessee, Texas. Florida, Tennessee, Texas. South Africa.

SECTION 10  
PAPAYA (*CARICA PAPAYA*)

1. Taxonomy and General Description

A. Taxonomy

Papaya, *Carica papaya* L., is an almost herbaceous (succulently soft-wooded), typically unbranched small tree in the family Caricaceae. Europeans first encountered papaya in the Western Hemisphere tropics by at least the early 1500s (Sauer, 1966), and various interests were soon disseminating it widely (Ferrão, 1992). Papaya is now cultivated worldwide in tropical and subtropical climates mainly for its melon-like fruit.

The Caricaceae is classified in the order Brassicales (sometimes called Capparales), which characteristically express mustard-oil glucosides (glucosinolates) (Jørgensen, 1995; Rodman *et al.*, 1998; Olson, 2002). Recently, consensus has been developing that the genus *Carica* L. has only the one species *C. papaya*, and that the Caricaceae may comprise six genera (Aradhyia *et al.*, 1999; Badillo, 2000; Van Droogenbroeck *et al.*, 2002, 2004; Kubitzki, 2003; Manshardt, 2002, Hawaii University, pers. com.). Most of the genera are neotropical forest plants, occurring in South America and Mesoamerica or only in Mesoamerica. *Vasconcellea*, the largest genus with 21 species, had usually been considered as a section within *Carica*. The other neotropical genera are *Jacaratia* (7 spp.), *Jarilla* (3 spp.) and *Horovitzia* (1 sp.) (Badillo, 1993). The sixth genus, *Cylicomorpha* (2 spp.), occurs in montane forests in equatorial Africa (Badillo, 1971).

The highland papayas, *Vasconcellea* (not "*Vasconcella*" – see Badillo, 2001; Kubitzki, 2003), are considered the closest relatives to *Carica papaya* (Badillo, 1993; Aradhyia *et al.*, 1999; Van Droogenbroeck *et al.*, 2002, 2004). *Vasconcellea* has many species with edible fruits (and a few cultivated varieties) (Badillo, 2000; Scheldeman and Van Damme, 2001); commercial cultivation of Caricaceae may be limited to the papaya and chamburo (ababai), babaco, and toronche or higacho (the names vary and sometimes are used locally for more than one species). The chamburo or mountain papaya, *V. cundinamarcensis* (often referred to as *V. pubescens*) is grown in the Americas; the fruits are usually cooked and eaten with sugar. There is commercial-scale cultivation in Chile, where the fruit is known as ababai (Scheldeman and Van Damme 2001). In western South America (particularly Ecuador) local consumers value babaco (often referred to as *V. pentagona*), which is also cultivated somewhat elsewhere, including New Zealand, South Africa, Spain and Italy (Scheldeman and Van Damme, 2001; Villarreal *et al.*, 2003). Babaco is generally considered to be an F<sub>1</sub> hybrid (known as *V. xheilbornii* but sometimes still as its var. *pentagona*) (Jiménez *et al.*, 1999; Wiersema and León, 1999; Scheldeman and Van Damme, 2001; Morales Astudillo *et al.*, 2004). Higacho (or broadly toronche), considered the hybrid *V. xheilbornii* var. *chrysopetala* (sometimes referred to as *V. chrysopetala*) is also found in Ecuador, with a commercial variety also grown in New Zealand (NRC, 1989; Scheldeman and Van Damme, 2001).

The International Plant Genetic Resources Institute (IPGRI) recognises eight different edible fruits from this family. Users from Mexico to South America collect fruits from wild plants or semi-wild plants, or may grow a few (thus incipient domestication). Siglalón silvestre, *V. stipulata*, is a local food in southern Ecuador. Col de monte (*V. monoica*) of Ecuador, Peru and Bolivia has small fruits eaten raw or

cooked. Chungay or mito (*V. candicans*) is a familiar food in Peru (De Feo *et al.*, 1999). Papayuelo (*V. goudotiana*) of Colombia is small and apple-like. Other edible *Vasconcellea* include tapaculo (bonete, papayito) or mountain pawpaw, *V. cauliflora*, whose fruit pulp is processed in various ways before consumption (Coppens d'Eeckenbrugge and Libreros Ferla, 2000), and higuera (calasacha), the collected nut *V. quercifolia*. Additionally, Scheldeman and Van Damme (2001) note for their edible fruits *V. crassipetala*, *V. microcarpa* (lechocillo), *V. palandensis* (papaïllo), *V. parviflora* (coral) and *V. sphaerocarpa* (higuillo negro). The fruits of *Jacaratia digitata*, *J. mexicana*, *J. spinosa*, and *Jarilla heterophylla* also are eaten locally (Whitmore, 1978; Scheldeman and Van Damme, 2001).

## B. Morphology

*Carica papaya* is a usually unbranched, giant-herb-like tufted tree 2-10 m in height; commercial producers often remove plants if they are reaching a height from which fruits would not be harvested easily. Large, palmately lobed leaves with long stout leaf-stalks (to 125 cm) are attached densely (alternating more or less spirally) near the terminus of the straight trunk, and spreading to form a loose open crown. The leaf-stalks (petioles) end in a leaf blade 20-60 cm (to 75-100 cm) across (Campostrini and Yamanishi, 2001a), with each blade usually 5- or 7-lobed, and each lobe cut pinnately. The trunk tapers from a 10-30 cm wide base to 5-7.5 cm at the crown, and is patterned conspicuously with large leaf-scars; it is thin-barked and often hollow (between nodes) with aging (Elias, 1980). The soft pulpy wood is formed predominantly by phloem, with little secondary xylem (Whitmore, 1978; Carlquist, 1998). This fast-growing plant has c. 15-30 mature leaves, with a leaf persisting 2.5-8 months and new leaves arising at the rate of 1.5 to nearly 4 per week (Sippel *et al.*, 1989; Allan *et al.*, 1997; Mabberley, 1998; Nakasone and Paull, 1998; Fournier *et al.*, 2003). Leaf senescence seems to be a function of the leaf's position within the plant's canopy (*i.e.* self-shading) rather than simply increasing age (Ackerly, 1999). All parts of the plant contain a thin, acrid latex, including the unripe fruits. The lifespan of feral trees is some 15-20 years (Anon, 2003). Plants infrequently may develop a forked trunk or a few branches when older or injured; in some places (*e.g.* Kenya) growers may encourage multiple trunks by pinching seedlings or cutting back established plants (Dodson and Gentry, 1978; Rao, 1993; Malo and Campbell, 1994).

The flower-bearing stalks arise in leaf axils. There are three basic flower types in domesticated plants, but with a range of possible variation, resulting overall in about six distinctive kinds (Storey, 1941; 1967; Hsu, 1958a, 1958b; Mosqueda Vázquez and Molina Galán, 1973; Fisher, 1980; Nakasone and Lamoureux, 1982):

- Female flowers (globose-ellipsoid ovary) (sometimes agronomically termed as “type 1”).
- Male flowers (of two kinds): morphologically typical (10 stamens, tiny rudimentary pistil) (“type 5”), or functionally male but a somewhat bisexual appearance (“type 4+”).
- Bisexual flowers (of three kinds): decandrous (10 stamens, elongate ovary) (“type 4”), pentandrous (5 stamens, deep-furrowed ± ovoid ovary) (“type 2”), or irregular (“type 3”) with stamens variably becoming carpel-like (*i.e.* carpellody).

The morphology of inflorescences and flowers varies with the sex of the tree. Varieties typically are either dioecious (with unisexual flowers and exclusively male and female plants), or they are polygamous (with bisexual and unisexual flowers and hermaphrodite and single-sex plants). On female plants, the stalk (peduncle) is just 2.5-6 cm long and has one or a few large bell-shaped flowers with curvy separate petals. On male plants, hanging branching stalks (panicles) 60-100 cm (to over 150 cm) long have many much smaller trumpet-shaped flowers, with the petals (and stamens' filaments) joined in a long narrow tube which has flared lobes (Fisher, 1980; Calif. Rare Fruit Growers, 1997; Nakasone and Paull, 1998; Ronse Decraene and Smets, 1999). On hermaphrodite plants, these structures are intermediate to the unisexual

types, with stalks less than 25 cm long having bisexual flowers that are shortly tubular with a midpoint or lower constriction and the petal lobes larger. Hermaphrodite plants sometimes also bear male flowers (Crop Knowl. Master, 1993). Some plants produce male flowers on short stalks.

The species thus has a richly complex capability in sexual expression and flowering. Some agricultural varieties (e.g. Solo and Eksotika) are gynodioecious — their trees are either hermaphrodite or female. Moreover, papaya sexuality can be labile. Bisexual flowers can be influenced by environmental conditions to change to male flowers through reductions in ovary size and function. Male (staminate) plants and andromonoecious plants (with male and hermaphrodite flowers) are phenotypically stable, or mutable. These types may exhibit seasonal sex reversal, developing staminate, bisexual, and pistillate flowers (Storey, 1958, 1976). Young hermaphrodite plants may have male flowers when first flowering, but bisexual flowers with maturity (Stambaugh, 1939). Female (pistillate) plants have stable sex expression; they are not known to develop flowers with masculine structures (Hofmeyr, 1939b; Nakasone and Paull, 1998). For details on conditions that may alter sexual expression and morphology, see Section V.D. Induced alterations to sexual development.

Fruits hang from the stalks attached to the upper trunk, below the old leaves, with the younger fruits above those more mature. Fruit shape is a consequence of selection for the preferences of various local users and markets, but also reflects the flower type; the generally large fruits vary from spherical or ovoid to pear-shaped or elongate, and 10-50 cm in length (Storey, 1969, 1987). Fruit weight can vary substantially (c. 0.35-10 kg or even 12 kg) (Font Quer, 1958; Linnell and Arnould, n.d.), again chiefly dependent on selection by local users and for specific markets. Storey (1969, 1987) reported preference for 2.5-6.0 kg fruits in South America and the South Pacific, 1.25-2.5 kg lobular fruits in South Africa, and just 400-500 g for Solo-type fruits (which were developed in Hawaii). For additional information on varieties and cultivars, see Table 1.22, and Section V.A. Reproductive types and locus of cultivation.

## 2. Distribution and Centre of Origin

*Carica papaya* is native in the north-tropical Western Hemisphere. Some have suggested a centre of origin in Central America or the south of Mexico (de Candolle, 1883, 1884, from Singh, 1990; Storey, 1976). Manshardt and Zee (1994) found wild papayas (exclusively dioecious) in the Caribbean coastal lowlands of southern Mexico and northern Honduras. The wild female plants produced golf ball-sized fruits of less than 100 g, which usually were inedible (Manshardt, 1999). The musty bitter fruits (berries) have an extensive investment in seeds, which are about 25% smaller than domesticated papaya seeds and have stronger requirements for breaking dormancy. In experimental testing, wild seeds needed strong light to germinate, but 75% of domesticated seeds germinated in darkness. Also, fluctuating temperatures partially inhibited wild seeds from germinating whereas variable temperature did not affect the domesticated seeds' germination (Paz and Vázquez-Yanes, 1998). On the Caribbean coast of Central America, feral papaya have traits apparently indicating greater introgression from wild papaya compared to the feral papaya on the Pacific coast, which appear to have fewer wild traits (Manshardt and Zee, 1994). Successively greater introgression of domestication traits in the wild plants along with increasing presence of feral domesticated-type plants has been found westward and southward from the known wild papaya region.

The centre of diversity for the relatively large genus *Vasconcellea* (formerly in *Carica*) is South America along the Andes, especially in Ecuador (Badillo, 1993; Morales Astudillo, *et al.*, 2004), with outlying species reaching as far as Mexico, Chile, Argentina and Uruguay (Aradhya *et al.*, 1999; Van Droogenbroeck *et al.*, 2004). This led some to propose South America for the origin for *C. papaya* (Prance, 1984). However, evidence to the contrary is provided by finding only domesticated-type feral *C. papaya* there (Manshardt and Zee, 1994; Morshidi, 1996), but finding unambiguously wild plants in Mexico and Honduras (Moreno, 1980; Manshardt and Zee, 1994; Paz and Vázquez-Yanes, 1998; Manshardt, 1999).

Furthermore, both isozyme and RAPD (random amplified polymorphic DNA) analyses, and RFLP (restriction fragment length polymorphism) analysis of chloroplast and mitochondrial intergenic sequences, show appreciable divergence of *C. papaya* from what is now recognised as the genus *Vasconcellea* (Jobin-Decor *et al.*, 1997; Aradhya *et al.*, 1999; Kim *et al.*, 2002; Van Droogenbroeck *et al.*, 2004). This correlates well with the experimental difficulty in forming hybrids of *C. papaya* with other species in Caricaceae (see Section VI.A. Interspecific crosses with *Carica papaya*). Because *C. papaya* is genetically so distinct, and only feral papaya are known in South America, nowadays a South American origin of wild papaya appears hardly tenable.

Papaya was probably domesticated in northern tropical America but a precise region has not been determined (Schroeder, 1958). Feral papayas occur in many tropical habitats of North America, Central America, the Caribbean and South America. In North America, subtropical areas of Mexico and Florida (USA) are the northernmost part of the species' current range; the southern range extends from Colombia and Venezuela to French Guiana, Brazil, Peru, Bolivia and Paraguay. In southern Florida there is evidence indicating pre-Columbian use of papaya by native people (Allen *et al.*, 2002). The Spanish and Portuguese encountered cultivated papaya on the Caribbean coasts of Mexico (being used by the Maya) and Panama and Colombia reportedly by at least 1519 and 1526, respectively; cultivated papaya was reported in Jamaica by at least 1756 (Sauer, 1966; Singh, 1990). In the 1500s papaya was transported to the Philippines and India, and it was readily disseminated into tropical Asia, Africa, and Pacific islands (Singh, 1990; Ferrão, 1992). Less widely used names for the fruit, the tree, or both include pawaw, paw paw (paw-paw, pawpaw) (but *Asimina* of the Annonaceae is known as pawpaw), papaye, papayer, papayo, lechosa, fruta bomba, melón zapote, mamón, mamonero, mamão and mamoeiro. *Carica papaya* is now well integrated into indigenous culture, agriculture and cuisine in numerous countries, and occurs beyond the cultivated areas as a feral or adventive plant, naturalised to various degrees in the tropics of the New and Old Worlds including Oceania.

### 3. Use as a Crop, and Agronomic Practices

Extensive non-commercial production of papaya is common, and much of the harvest in some countries is not exported. Instead, growers consume the fruits or trade them locally. For example, Indonesia estimated that it produced 744,000 tons, of which less than 4 tons were exported (Setyobudi and Purnomo, 1999). In Vietnam, 50% of farm households cultivate papaya in home gardens, with 5 to 10 million or more growing 1-10 trees, whereas only 5,000-10,000 farmers produce papaya in monoculture gardens or large fields (Le Tran and Tran, 1999). In the Philippines, as many as 1.5 million farmers derive cash income from the sale of papaya from home garden, monoculture, or multiple-cropping system agriculture (Kositratana *et al.*, 1999). Income from a unit of land in papaya cultivation may be two to four times more than the income from rice; papaya's entire value to the small farmer should not be underestimated (The Papaya Biotechnology Network of Southeast Asia-Workshop participants, 1999; ISAAA; cf. Cook, 2004).

The commercially reported production of papaya in 52 countries in 2004 reached 6.5 million metric tons (FAO, 2005). The total area harvested was 365, 846 ha. By region, 7 of the production areas are in Middle America (Central America plus North America), 5 in the Caribbean, 10 in South America, 11 in Africa, 4 in the Near East, 10 in Asia plus Australia, and 5 in Oceania. The major producers were especially Brazil (24.6%), Mexico, Nigeria, India and Indonesia, as well as Ethiopia, the Democratic Republic of the Congo, Peru, Venezuela and China.

## A. Uses, and adverse effects

### *Industrial uses*

Papaya is primarily a fresh-market fruit, and is used in drinks, jams, pectin, candies and as crystallised fruit. Green fruit may be cooked as a vegetable, as may the leaves, flowers and roots (Duke, 1967; Watson, 1997). Papaya has several well-known industrial uses, notably for the enzyme papain (one of its four major constituent cysteine proteinases) (El Moussaoui *et al.*, 2001), which has properties similar to gastric pepsin. Producers induce latex to exude from longitudinal incisions made into unripe fruit; the papain purified from the extract is used in foods, beverages, pharmaceuticals, and other manufacturing (Mabberley, 1998; Wiersema and León, 1999). For example, the food industry uses papain in brewing, manufacturing baby food, and producing proteins for human and animal consumption. Papain is also used to shrinkproof wool and silk, and in the bating process to make leathers more pliable. For some applications however, synthetic enzymes and enzymes from other sources are displacing the use of the natural papain (Watson, 1997; ETA, 2001). The latex from papaya has been used in manufacture of chewing gum (de Wit, 1966). Oil from the fruit's many (200-1000) more or less spheroidal seeds (c. 2-5 mm × 3.5-6 mm) (Sharma and Singh, 1975), and other components of fruit and leaves have been used in cosmetics and soap (Quenum, 2001).

### *Nutritional and medical uses*

Papaya constituents contribute to human nutrition and health. Vitamins A and C from one medium papaya (edible portion 350 g) exceed the Dietary Reference Intakes established by the U.S. Food and Nutrition Board (Inst. Medicine, Natl. Acad. Sci.) for adult minimum daily requirements (CRN, 2001; USDA, 2001), and papaya is a good source of the minerals K, Mg and B (Hardisson *et al.*, 2001). Papaya has traditional and modern medical and dental uses; fruits, seeds, latex, and extracts have been used for treating at least forty human conditions, and are being investigated for others (e.g. Lewis and Elvin-Lewis, 1977; Mezhlumyan *et al.*, 2003; Petitto, 2004). The efficacy of some of the uses is well documented (Animal Sci. Dept. Cornell Univ., 2001), including those as an anthelmintic (Satrija *et al.*, 1995); an antiamoebic (To and Kyu, 1934), possibly mediated by the alkaloid carpaine (Burdick, 1971); and an enterobacteria antimicrobial (Osato *et al.*, 1993). Papain is used in preparation or manufacturing of adjuvants and reagents for antibiotics or vaccines; chymopapain is a biologic used for treatment of herniated disks in the spine (Quenum, 2001; Mezhlumyan *et al.*, 2003).

### *Adverse and other consequences*

Hypersensitive or allergic human responses to papaya have been described, including respiratory responses to the pollen (Blanco *et al.*, 1998). Consumption of ripe fruit only infrequently produces such adverse consequences (Castillo *et al.*, 1996; Ilicv and Elsner, 1997). Skin may have such responses to fruit contact (Ezeoke, 1985) or extracts (Banik *et al.*, 1992). Contact with latex derived from abraded green fruits and plant parts or extracts that contain papain or other proteinases may harm unprotected skin, but can also be used in healing wounds (Mezhlumyan *et al.*, 2003). Tissues of papaya (including leaves and roots) which contain cyanogenic glycosides (Olafsdottir *et al.*, 2002; Seigler *et al.*, 2002) and tannins may provoke adverse reactions if consumed in quantity.

Papaya enzymes may be injected for medical purposes. However, Moneret-Vautrain *et al.* (1985) have described the allergenic potential of injected chymopapain extracts — up to 1% of the population may have an adverse reaction. Injection may also evoke immune responses to papaya's other known cysteine proteinases, *i.e.* papain, caricain, and glycyl endopeptidase (Dando *et al.*, 1995). The reactions to the fruit, pollen, and papain are mediated by an IgE mechanism (Blanco *et al.*, 1998; Soto-Mera *et al.*, 2000).

Papaya has been investigated for possible effects on pregnancy, since some consider consumption a risk to fetal development, or to cause or alternatively to prevent miscarriage (Eno *et al.*, 2000; Adebisi *et al.*, 2002a). In a controlled study of pregnant rats, juice from ripe fruits was considered safe (Adebisi *et al.*, 2002a). No effect of papaya juice was observed on isolated uterine muscle *in vitro*, but crude latex preparations caused spasm (Adebisi *et al.*, 2002a, 2002b). Additional physiological effects have been described from papaya parts not usually consumed, as for example on reproduction in male rodents and monkeys. Mice, rats and Hanuman langur monkeys (*Semnopithecus entellus*) given extracts of papaya seed appear to become infertile reversibly without indication of toxicity (Chinoy *et al.*, 1994; Pathak *et al.*, 2000; Lohiya *et al.*, 2002). A papaya seed extract may offer control of a protozoan parasite that causes a major disease of fish in aquaculture (Ekanem *et al.*, 2004).

## **B. Propagation**

### *Seed*

Papaya producers usually grow the crop from seed; agronomic advisers encourage purchasing commercial seed for propagation (Muthukrishnan and Irulappan, 1985). Most commercial seed producers offer inbred selections, but some hybrid lines such as Rainbow and Eksotika II are available. Further discussion on the place of hybrid seed is found below in this section's subsection B.3. Varietal selection, and in Section VII.B.2. Cultivated varieties as sources of genetic variability. A list of frequently encountered varieties in commercial and breeding use is in Table 1.22.

To assure seed quality, growers must take into account the sexual reproductive type and the genetic variability of the variety. Lines heterogeneous for many traits will produce considerable trait diversity in the progeny. Dioecious varieties are open-pollinated and much phenotypic variability can arise in the seeds of on-site fruit-bearing trees. Dioecious lines (described in Section V.A. Reproductive types and locus of cultivation) are more likely to be heterozygous for quality traits; the characteristics of tree and fruit are only maintained exceptionally. To maintain traits for generations, producers strictly control pollination using standard breeding techniques, and careful isolation of multiple lines. Rigorous crossing procedures are preferable (Watson, 1997). A strategy available for maintaining the consistency of some varieties is to obtain seed only from selfed male trees ("ambivalent males"), which produce fruit under limited (such as seasonal) circumstances (Aquilizan, 1987). Traits of superior plants can also be exploited by selection, especially when growers bag and hand-pollinate flowers to produce the seed.

In the hermaphrodite lines self-pollination predominates, but outcrossing is not excluded (see also Section V.C. Pollination). Selection of seeds from only-selfed hermaphrodite plants will provide better trait uniformity (Singh, 1990). Carefully controlled production of hybrid seed is an alternative that may be increasing in importance. Seed producers pollinate selected female trees using pollen from selected hermaphrodite trees.

Seeds, including the outermost layer — a gelatinous sarcotesta (Fisher, 1980), have inhibitors that prevent germination while contained in the fruit or prematurely after release (Yahiro and Hayashi, 1982; Ellis *et al.*, 1985; Arumugum and Shanmugavelu, 1975; Tseng, 1992). Seeds freshly harvested from the fruits have very low and variable germination. Removal of the sarcotesta considerably increases germination in the fresh undried seeds. Seed treatment by drying and cool storage and soaking prior to planting can promote viability and the rate and uniformity of germination. Storage below 15°C for 30-50 days greatly reduces the activity of growth inhibitors and enhances germination (Yahiro 1979; Yahiro and Hayashi, 1982). Soaking (with changes of water) also greatly increases germination (Paz and Vázquez-Yanes, 1998). For improved long-term storage, seed can be dried to moisture levels of 9-12% (Teng and Hor, 1976; Ellis *et al.*, 1991); if dry and cool, papaya seeds may retain viability for 3 years (Malo and Campbell, 1994). After desiccation, heat shock can break the dormancy (Wood *et al.*, 2000).

Papaya producers either sow seed in the field, or start by germinating seed in a nursery. Germination may occur in 10-21 days after sowing, or in 4-10 days after pretreatment, and may continue intermittently for up to 35-40 days (Chen and Tseng, 1996; Bhattacharya and Khuspe, 2001). Nursery-grown seedlings may be transplanted to the field at about 60 days (Muthukrishnan and Irulappan, 1985). Because the sex of a plant is not known until flowering, growers often plant additional papaya in each mound, and later thin plants of the undesired sex. If growing hermaphrodite plants is intended, two to four (gynodioecious-type) plants may be transplanted per mound. Growers then remove the female saplings when the sex can be determined — in *c.* 4-8 months from sowing. If dioecious plants are grown, then surplus males will be discarded, leaving one male tree to pollinate 10-15 (sometimes more) female trees.

However, several assays have recently been developed that will likely lead to routine molecular tests for determining the sex of seedlings. They include an assay for males and hermaphrodites using a sequence characterised amplified region (SCAR) marker developed from an RAPD marker (Urasaki *et al.*, 2002a, 2002b); a pair of SCAR markers whose products are not produced in females (Deputy *et al.*, 2002); a male-specific simple sequence repeat (SSR) (or microsatellite) and a SCAR marker (Parasnis *et al.*, 1999, 2000); and a hermaphrodite-specific RAPD probe (Lemos *et al.*, 2002). Moreover, Chan-Tai *et al.* (2003) are evaluating an exclusively hermaphrodite Sunrise Solo mutant that when selfed appears to be stable in its sexual phenotype, producing only hermaphrodites.

#### *Vegetative propagation*

The cultivar Hortus Gold of South Africa is propagated vegetatively using leafy stem cuttings (Allan, 1974). Experimental success in propagating papaya by cuttings was reported by Allan (1964). Large leafy lateral shoots that developed after a winter season, were initially used for the cuttings, and rooted under intermittent mist. Induction and proliferation of suitable-sized lateral shoots (breaking apical dominance) is improved with application of cytokinin and gibberellic acid mixtures to developed plants (Allan, 1995; Ono *et al.*, 2004). The cuttings root in about 3 weeks. Exceptionally, some varieties of papaya are seedless (Wettstein *et al.*, 1944; de Wit, 1966).

Clonally propagated plants may show greater uniformity, earlier fruiting, lower fruit-bearing height, and improved yield over plants from seed (Drew, 1988; Chan and Teo, 2002). Hawaiian Rainbow transgenic papaya propagated from cuttings flowered 1-3 months earlier and bore fruit 30 cm lower than progeny from seed (Fitch *et al.*, 2002). Cloned plants also yielded significantly greater fruit weight, a difference more marked under less favourable environments. The technology for small-scale commercial and experimental micropropagation is well developed (Litz and Conover, 1978; Drew, 1992; Magdalita *et al.*, 1997a). Field trials of *in vitro* plantlets have found that they propagate true to sex, without somaclonal reversion.

Papaya can also be propagated by grafting. Airi *et al.* (1986) cleft-grafted scion shoots from cultivars Co-1 and Honey Dew onto uniformly established seedlings. Patch and T budding also can be used, but the success rate is poorer than with cleft grafting. In Malaysia, some growers use grafting in the orchard to supersede female-fruited trees of the cultivar Eksotika (Cheah *et al.*, 1993). As soon as a plant's sex is determined, propagators will side-cleft graft the female trees with scion shoots (basal diameter 2-3 cm) taken from hermaphrodite Eksotika trees; the scions fruit 6 months after the field grafting.

#### *Varietal selection: Inbred lines and hybrid cultivars*

Although local papaya varieties are most common in some markets, producers frequently grow other varieties that originated elsewhere. Singh (1990) noted that in terms of phenotypic uniformity and stability, few varieties qualified strictly as cultivars, although Solo came closest. As described in Section VII.B.2. Cultivated varieties as sources of genetic variability, the Solo group of varieties developed in Hawaii has

limited but inherent genetic variability. Other such uniform lines are Eksotika and Eksotika II from Malaysia, and the Tainung series (Formosa group) of hybrids and inbreds distributed by the Known You seed company of Taiwan (Manshardt, 2002, pers. com.). Commercial producers often grow Solo varieties, Khaek Dam from Thailand, and Tainung (Subhadrabandhu and Nontaswatsri 1997; Le Tran and Tran, 1999; Story, 2001); other Solo cultivars include Kapoho and Waimanalo (Watson, 1997; Beltraide, 2000). Larger-type fruits such as Maradol are also planted extensively. Table 1.22 lists some common varieties used in commerce and breeding.

**Table 1.22 Common papaya varieties in commerce and breeding**

Variety	Origin	Average fruit size, Notable traits	Fruit characteristics (e.g. shape, color)
Bettina	Australia (Florida Betty × Queensland var.)	1.36-2.27 kg	Round-ovoid. Well-colored.
Cariflora	Florida, USA	0.8 kg Tolerant to PRSV.	Round. Dark yellow to light orange flesh.
Coorg Honey Dew <sup>H</sup>	India	2-3.5 kg	Long to ovoid. Yellow.
Eksotika <sup>H</sup>	Malaysia (Sunrise Solo × Subang 6)	0.6-0.9 kg	Elongate (from hermaphrodite). Orange-red flesh.
Eksotika II <sup>H</sup>	Malaysia (Eksotika lines 19 × 20)	0.6-1.0 kg Higher yield than Eksotika.	Fewer freckles on skin, and sweeter than Eksotika.
Sekaki <sup>H</sup>	Malaysia	1.0-2.5 kg	Long, cylindrical, with smooth skin. Red, firm flesh.
Hortus Gold (selection: Honey Gold)	South Africa	1 kg Propagated by cuttings.	Round-ovoid. Golden yellow.
Known You 1 <sup>H</sup>	Taiwan	1.6-3 kg Tolerant to PRSV.	Very long and slender. Yellow flesh.
Maradol	Cuba	2.6 kg	Elongate. Green or yellow skin.
Rainbow <sup>H</sup>	Hawaii, USA (SunUp × Kapoho Solo)	0.65 kg Transgenic resistance to PRSV.	Pear-shaped to ellipsoid. Yellow-orange flesh.
Red Lady 786	Taiwan	1.5-2 kg Tolerant to PRSV.	Elongate. Red flesh.
Red Maradol Solo <sup>H</sup>	Mexico Developed in Hawaii, USA; from Barbados originally.	2.5-2.6 kg 0.5-1 kg Bisexual flowers highly selfing.	Red flesh; yellow-orange skin. Pear-shaped (from hermaphrodites). Orange-yellow skin; golden orange flesh.
Kapoho Solo <sup>H</sup>	Hawaii, USA	0.45 kg	Pear-shaped, but shorter neck than Sunrise Solo. Orange-yellow flesh.
Sunrise Solo <sup>H</sup>	Hawaii, USA	0.57 kg	Pear-shaped. Reddish pink flesh.
Tainung 1 <sup>H</sup>	Taiwan	1.1 kg	Pointed blossom-end (from hermaphrodite). Red flesh.

<sup>H</sup> Hermaphrodite variety (i.e. gynodioecious)

Historically, papaya researchers have not found hybrid production necessary for improvement of the crop. More recently, however, hybridisation has demonstrably improved crop potential. The heterosis in  $F_1$  hybrids in some cases has increased plant vigor and yield in agronomic assessments (Subramanyam and Iyer, 1984; Dinesh *et al.*, 1992; Chan, 2001) and seed producer trials (Grant, G., 2004, Papaya Seed Australia, pers. com.). In Malaysia, hybridisation of Eksotika Line 20 with its sib-line produced the  $F_1$  hybrid Eksotika II, which has heterosis in vigor and yield (Chan, 1992). In Hawaii, the transgenic cultivar Rainbow was derived as the  $F_1$  progeny of gynodioecious parental lines SunUp (transgenic) and Kapoho (a popular nontransgenic). In Australia, about 65% of commercial plantings over 10 ha are the dioecious

Hybrid 1B (Grant, G., 2004, Papaya Seed Australia, pers. com.) and other hybrids are readily available from commercial sources and commonly planted. The Queensland government maintains parental varieties used for hybrid production and initially had a voluntary seed production scheme with a program for training hybrid seed producers (Dunn, J., 2004, Queensland Dept. Primary Indust. & Fisheries, pers. com.). In diallel crosses, desirable agronomic characters have shown good combining ability (Dinesh *et al.*, 1992; Subhadrabandhu and Nontaswatsri, 1997).

### C. Cropping practices

Trees begin bearing within the 1st year of planting (some varieties within 7-9 months). Commercial life of the plant in the large-scale commercial production cycle is usually 3 years, but may be less or more in some areas (Singh, 1990; Watson, 1997). In the U.S. Virgin Islands, early, low-bearing plants have been developed for an annual crop to minimise damage from papaya ringspot virus and seasonal hurricanes (Zimmerman and Kowalski, 2004). In Hawaii production in the 4th year drops off precipitously, thus encouraging replanting after the 3rd year (Younge and Plucknett, 1981). Continuous production is possible even in subtropical regions if winter temperatures may be moderated such as by ocean buffering, but flowering during cooler months in some locations may result in reduced summer harvests (Watson, 1997). Even though fruit production may occur throughout the year in many regions, the month of planting can have an impact on the plant's development, the timing of harvest, and the total yield (Singh and Singh, 1998). Fruit may begin to ripen within 7-9 months, and full-production harvesting may be possible within another 2 months. Depending upon variety, the fruits may need to be thinned. For example, Sunrise Solo may bear up to 5 fruits per node, but is reduced by hand to 2 fruits (Watson, 1997).

Density of planting depends upon the papaya variety and the region where cultivated; typical practice establishes 1160 to 1930 plants per ha (Watson, 1997), with trees spaced from 1.8 to 2.7 m apart in the row and a distance between rows of 2.7 to 3 m. Sometimes double rows are planted, *e.g.* 3.25 × 1.75 × 2.4 m (PROSEA, 1991). Successful practices include provision of optimal soil cover for the orchard space between trees. Younge and Plucknett (1981) showed that clover (*e.g.* *Trifolium*) or grass reduces return compared to clean cultivation; weedy coverage of the space is similarly disadvantageous. Trash mulching may improve yields, as may a year of rotation with clover between several continuous years of papaya production. Mulching with coarse grass hay may substantially increase yields (Elder *et al.*, 2002a).

Papaya can be intercropped if timed appropriately. Before the papaya trees reach bearing age in India, short-term vegetable cropping may be accomplished with tomatoes (*Lycopersicon*), onions (*Allium*), or cabbage or cauliflower (*Brassica*) (Muthukrishnan and Irulappan, 1985), but it was recommended that any competition to the papayas be eliminated during papaya's many months of fruiting to avoid reducing yield. In Nigeria intercropping has been tested with jute (*Corchorus*), sweet potato (*Ipomoea batatas*), okra (*Abelmoschus esculentus*), and watermelon (*Citrullus lanatus*) (Aiyelaagbe and Jolaoso, 1992). However, the cotton or melon aphid (*Aphis gossypii*) can transmit papaya ringspot virus from cucurbits (Cucurbitaceae) (Ali *et al.*, 2004). Some growers apparently use papaya itself as a short-term intercrop between rows of mangos (*Mangifera indica*) or litchis (*Litchi chinensis*) (Muthukrishnan and Irulappan, 1985). In Malaysia, sometimes papaya is the intercrop with oil palm (*Elaeis guineensis*) or rubber (*Hevea brasiliensis*) on developing plantations (Chan *et al.*, 1999). As papaya is often a 2- or 3-year crop, such interplantings have little impact on the longer lived trees before they reach production age.

### D. Cultivation requirements

Adequate irrigation is essential for plant growth and fruit quality. Rainfall in the 1000 to 2500 mm range may be optimal (Watson, 1997), and seasonal variability needs to be taken into account. The pattern of rainfall may lead to soil water deficits, so that supplemental water must be provided for fruit production (Terra de Almeida *et al.*, 2003b). Practices include overhead or modified drip or undertree irrigation.

Overhead irrigation may be least suitable, since leaf diseases may increase (Watson, 1997). For Australia, Watson (1997) recommended recharging the root zone twice a week to attain soil water capacity. A pH of 5.0-7.0 is favorable for papaya cultivation (Nakasone and Paull, 1998); lime is commonly used to increase alkalinity.

Balanced nutrition should be provided from the date of planting through harvesting for this fast-growing, heavy-bearing crop (Cunha and Haag, 1980; Watson, 1997). Nitrogen, phosphorus and potassium are important for good growth (Shoji *et al.*, 1958; Awada *et al.*, 1986; Nakasone and Paull, 1998). Nitrogen deficiency coupled with declining air temperatures can produce carpellodic flowers in some varieties and unmarketable fruits (Awada and Ikeda, 1957). Boron deficiency causes “bumpy” fruit and latex exudation (Chan and Raveendranathan, 1984), which can be remedied by foliar spray of boric acid or ground application of borax.

#### E. Yield parameters

The yield of papaya fruit varies widely in different countries, and is dependent on soil characteristics, varieties grown, pest and pathogen incidence, and local good management practices (Singh, 1990). Productivity of an orchard is also a function of the number of bearing trees; hermaphrodite and female plant types; average number of fruits per tree, which may be 25-100; and average weight of fruit, which commonly ranges between 350 g and 3000 g. The average yield worldwide in weight of fruit for the 10 years of 1991-2000 was 15,000 kg per ha (FAO, 2001). Optimal production is between 27,000 to 35,000 kg per ha in India (Singh, 1990); 12,500 to 62,500 kg per ha in Trinidad; and experimentally up to 100,000 kg per ha in Hawaii, although the typical Hawaiian yields are 20,000 to 30,000 kg per ha (excluding culled fruit) (Manshardt, 2002, pers. com.). Yield over the lifetime of the tree varies; with intensive cultivation, the highest yield is in the 1<sup>st</sup> year following planting.

Several thousand metric tons of papaya latex are obtained each year from the unripe fruits. An orchard of not less than 10 ha is usually required to produce one metric ton of dry latex annually (El Moussaoui *et al.*, 2001).

Papaya seed production for agriculture varies greatly with variety, growing conditions, cultivation practices (*e.g.* open-pollination in isolated fields, or controlled hand-pollination), and purpose (*e.g.* production of foundation seed for the market, or breeding seed). In India (Bihar) for example, the dioecious Pusa Dwarf and Pusa Giant produced more seeds at lower cost under hand-pollination than the gynodioecious Pusa Delicious and Pusa Majesty. The seed yield of Pusa Dwarf in isolation plots at one site was 579 kg/ha, whereas under controlled pollination at another site the yield was 362 kg/ha. The yield of hand-pollinated Pusa Majesty was just 52 kg/ha (Ram and Majumdar, 1990; Ram, 1996).

#### 4. Pests and Pathogens

*Carica papaya*'s milky latex is stored in a dense network of anastomosing articulated laticifers (joined cells) throughout the plant, but not within the fruit when ripe (Roth and Clausnitzer, 1972; Fisher, 1980; Zeng *et al.*, 1994). The latex may provide defense mechanisms by sanitising and healing wounds. The soluble fraction of the latex (which is *c.* 85% water) contains a rich diversity of biomolecules, including some possibly involved or recognised to be directly involved in deterring insects or pathogens — such as glycosyl hydrolases (*e.g.* a class II papaya chitinase), proteinase inhibitors (papaya cystatins), and nine proteinases (El Moussaoui *et al.*, 2001; Azarkan *et al.*, 2004). Papain (which is not papaya's major cysteine proteinase) is a crucial factor in defense against some lepidopteran larvae (*Samia*, Saturniidae; and *Mamestra* and *Spodoptera*, Noctuidae) (Konno *et al.*, 2004). Nonetheless, papaya seedlings experimentally exposed to key damaging mite species (*Tetranychus* and *Calacarus*) and the powdery mildew fungus *Oidium caricae* did not show induced resistance, but instead weak induced susceptibility after being

sanitised and transplanted to the field in Hawaii (Fournier *et al.*, 2004). Major pests and pathogens evade defenses, which can also enhance infestation by other such species.

#### A. Pest species: Mites, insects and nematodes

A small number of mite and insect species are major pests of papaya, though many such species infest the plants (Singh, 1990; Pantoja *et al.*, 2002). Aphids often increase in number on weeds surrounding or within the orchards and when the weeds have dried up, attack papaya (Singh, 1990). Green peach aphid (*Myzus persicae*), cotton or melon aphid (*Aphis gossypii*) and cowpea aphid (*A. craccivora*) may transmit papaya ringspot virus. Other insect pests include onion thrips (*Thrips tabaci*) in Hawaii; various scale insects, such as *Aonidiella orientalis* in Queensland; and mealybugs (Pseudococcidae). Fruit flies, such as oriental fruit fly (*Bactrocera dorsalis*) and Mediterranean fruit fly (*Ceratitis capitata*), are principal infesting species in Hawaii (Manashardt, 2002, pers. com.), as are papaya fruit fly (*Toxotrypana curvicauda*) in the Caribbean region (Malo and Campbell, 1994); fruit flies are of most importance in the export market (Nakasone and Paull, 1998). Leafhoppers can be significant pests, including *Empoasca papayae* in Brazil (Firko and Podleckis, 1996) and *E. stevensii* in Hawaii (Manashardt, 2002, pers. com.). Most insect infestations can be treated with appropriate pesticides; aphids may be controlled best by prophylactic removal of host weeds in the vicinity of the orchards (Singh, 1990).

Mite species are likely to be more important pests than insects, with false spider mites (*e.g. Brevipalpus phoenicis*) and spider mites (*e.g. carmine mite, Tetranychus cinnabarinus*) occurring in most growing areas (Singh, 1990). Tarsonemid mites (*e.g. Polyphagotarsonemus latus*) are pests in Brazil (Firko and Podleckis, 1996). Suitable acaricides may be employed to control mite damage (Singh, 1990). Root knot nematodes (*Meloidogyne* spp.) and reniform nematode (*Rotylenchulus reniformis*) may limit production in some countries (Singh, 1990). Producers use various halogenated soil fumigants to control nematodes, along with cultural strategies such as removing papaya debris and rotating papaya with other crops.

#### B. Pathogens: Fungi, viruses and bacteria

More important than mite and insect pests are the pathogens that infect various developmental stages and parts of the plant. The severity of infection depends upon cultural practices and environmental conditions. Singh (1990) reported up to seventeen papaya diseases, of which about six are principal diseases found in many growing areas. Many of the areas are affected by collar rots, damping off, anthracnose, mosaic, and leaf curl diseases. Nishijima (1999) lists a larger number of papaya disease organisms and the associated disease conditions; a recent description of the principal diseases is provided by Persley and Ploetz (2003).

Several fungi produce damping off diseases in nursery plants (younger than 60 days following seeding); the causal agents are *Phytophthora*, *Pythium* and *Rhizoctonia* species. Collar rots are important diseases, affecting seedlings and older plants (stems rot and crack, leading to death); *Pythium* and *Phytophthora* along with *Calonectria* are the causal agents. An anthracnose is in most areas, infesting leaf petioles and fruits; *Colletotrichum gloeosporioides* causes this major disease. Fungal infections of leaf blades and petioles include *Corynespora* leaf spot, which may be caused by *Corynespora cassicola* (Caribbean region). Powdery mildews affecting the fruit or other parts include *Oidium caricae* (Hawaii), *Sphaerotheca* spp. and *Leveillula taurica* (*Oidiopsis taurica*) (Queensland), and *Ovulariopsis papayae* (East Africa) (Morton, 1987).

Postharvest fungal diseases also cause losses. *Phytophthora* stem-end rot (*Phytophthora nicotianae* var. *parasitica*), *Phomopsis* rot (*Phomopsis caricae-papayae*), anthracnose (*C. gloeosporioides*), black stem-end rot (*Phoma caricae-papayae* and *Lasiodiplodia theobromae*) and *Alternaria* rot (*Alternaria*

*alternata*) may follow inadequate temperature maintenance or mechanical injury (Kader, 2000). Anthracnoses and Phytophthora blights may be controlled by various fungicides (Pernezny and Litz, 1999). Unspecified replant or yield decline problems (which are probably caused by fungal pathogens) have been treated by replacement of infected soil with virgin soil, or by fumigation (CTAHR, 1985).

Viruses usually impose the most significant limits to papaya cultivation; the importance of some viruses seems to be increasing in many growing areas. Papaya ringspot virus (PRSV), a potyvirus, has produced major crop loss, for example in Hawaii, Mexico, the Caribbean, South America, Africa and Southeast Asia (Persley and Ploetz, 2003). Diagnostic are dark green rings on fruit, and yellow mosaic on leaf lamina accompanied by stunting and shoestring-like leaves (Gonsalves, 1993). PRSV is spread by mechanical means, and also green peach aphid and cotton or melon aphid (Bhargava and Khurana, 1970). Declines in production may occur despite vigorous efforts using cultural strategies to limit spread. Control strategies have included roguing infected plants, but this cannot stem the disease once established (Queensland Dept. Primary Indust. & Fisheries 2003). Treatment with aphicides has been ineffective, because transmission by insertion into the plant occurs before the aphids are killed (Pernezny and Litz, 1999). If non-host crops are interplanted between papaya rows, vectors feed on the non-hosts before feeding on papayas, which can reduce disease transmission and incidence (Gonsalves, 1998). Researchers in Taiwan have developed some tolerant varieties. Seedlings were inoculated with a mild strain of PRSV or a mutated virus to produce cross protection from the more devastating form (Yeh, 1990). However, because of the apparent mutability of the virus, this protection is not completely effective and may not be permanent (Lin *et al.*, 1989). Also, the present tolerant varieties (*e.g.* Tainung No. 5) have had poor acceptance because of inferior consumer qualities (Japan Intl. Res. Centre Agric. Sci., 2003).

Collaborators in Cornell University and Hawaii developed transgenic plants to provide resistance to PRSV by expression of viral coat proteins. The newly introduced cultivars brought about a rapid reversal of decline in the papaya industry in Hawaii (Lius *et al.*, 1997; Manshardt, 1999; Gonsalves, 2000). Small field trials were conducted in 1992; a scale-up and the release of seeds commercially were completed in 1998. In 2003, nearly half the commercial crop in Hawaii consisted of the PRSV-resistant Rainbow transgenic papaya (Pacific Bus. News 2003). For details about the coat protein-based resistance, see Section VII.B.4. Molecular approaches for agronomic improvement.

Other viruses include papaya mosaic virus (PapMV), a potexvirus identified in parts of South America and Florida (USA) (Malo and Campbell, 1994). PapMV appears to be mechanically transmitted, without a biological vector (Buchen-Osmond and Hiebert, 1988). The incompletely characterised papaya leaf-distortion mosaic virus (PLDMV), a potyvirus, has been analysed experimentally (Maoka *et al.*, 1996); it has no more than 59% amino acid sequence homology to PRSV. Chen *et al.* (2001) have shown that the Taiwan isolate DL-1 is antigenically different from PRSV; this virus is also known in Japan (Maoka, 2002). Papaya droopy necrosis virus (PDNV), a rhabdovirus, is found in Florida (Zettler and Wan, 1993). A similar rhabdovirus called papaya apical necrosis virus ("PANV") (but not recognised by the International Committee on the Taxonomy of Viruses) is often a severe pathogen in Venezuela (Zettler and Wan, 1993); it is vectored by a leafhopper (Lastra and Quintero, 1981). Croton yellow vein mosaic virus (CYVMV), a bigeminivirus, causes severe leaf curling and twisting of petioles, leading to death before flowering or fruiting (Singh, 1990; Brunt *et al.*, 1996). This can be a devastating pathogen in some areas, such as India; it appears to be transmitted by the whitefly *Bemisia tabaci* even though papaya is not a preferred host. Papaya leaf curl virus (PLCV), a whitefly-transmitted geminivirus, is found in India and Taiwan (Chang *et al.*, 2003); it has been analysed for molecular similarity to other viruses (Saxena *et al.*, 1998). Papaya lethal yellowing virus (PLYV), with substantial sequence similarity to *Tombusvirus* (Silva *et al.*, 1997), can be economically important in Brazil. Tomato spotted wilt virus (TSWV), a tospovirus, has caused sporadic outbreaks in Hawaii; it is hosted by common weed species and vectored by thrips (Gonsalves and Trujillo, 1986; Bautista *et al.*, 1995). PRSV type P (papaya) infects papaya and cucurbits; PRSV type W (watermelon) naturally only infects cucurbits, but experimentally it has infected papaya

(Purcifull *et al.*, 1986). Also detected have been diseases attributed to distortion ringspot virus, which may actually be a synonym for PRSV (Brunt *et al.*, 1996). A regionally important virus in Brazil called papaya meleira virus (“PMeV”) (which is currently not ICTV-sanctioned) is a double-stranded DNA virus with no similarities to other viruses (Maciel-Zambolim *et al.*, 2003). Control strategies for the other virus diseases of papaya are similar to those for PRSV, but resistance strategies have not yet been developed.

Multiple viruses or other pathogens may occur as coextensive diseases in papaya-growing regions, as do PLYV and PRSV in Venezuela (Marys *et al.*, 2000). Evidence for coinfection by PapMV and PRSV has been found in one of ten Mexican states that were assessed (Noa-Carrazana *et al.*, 2000). Simultaneous occurrences of zucchini yellow mosaic virus (ZYMV), PRSV, and papaya bunchy top disease have also been detected (Fewerda-Licha, 2002).

Bacterial diseases of papaya are more or less common depending upon the growing region. Species of *Erwinia* initially induce lesions on the lower surface of leaves; yellowing, wilting and death of foliage occur and rotting of the plant follows (Seaver, 2000). This disease is more economically significant in the Caribbean and Venezuela than PRSV (Coppens d’Eeckenbrugge, G, IPGRI Cali, Colombia, pers. com.). To control *Erwinia* in the Virgin Islands, Webb (1985) recommended resistant cultivars and barrier crops that did not support the pathogen, as bactericides and antibiotics were not effective. In the Northern Mariana Islands, disease caused by *Erwinia* is spread by the giant African snail *Achatina fulica* and disease incidence is reduced by snail control.

A severe dieback of papaya in Australia had been attributed to *Phytoplasma australiense* (Liefiting *et al.*, 1998). Two diseases were identified, papaya yellow crinkle disease and papaya dieback disease, caused by two different phytoplasma groups (Padovan and Gibb, 2001). A small incidence of a third phytoplasma disease in Australia, a papaya mosaic disease, has been reported (Elder *et al.*, 2002b). Leafhoppers (*e.g.* *Orosius*) infrequently harbored the phytoplasmas, suggesting transmittal but the attribution for causation of the disease is qualified because papaya is not a preferred host. Papaya bunchy top disease appears to be produced by rickettsial bacteria in the laticifers, which are transmitted by the leafhopper *Empoasca papayae* (Davis *et al.*, 1998, 1999). Control strategies for bunchy top have included roguing infected plants, topping to allow production of uninfected axillary shoots, and control of vectoring aphids (Davis, 1993).

## 5. Reproductive Biology

### A. Reproductive types and locus of cultivation

Papayas may be cultivated as dioecious varieties (with separate male and female plants), or gynodioecious varieties (having both hermaphrodite and female plants). In subtropical areas dioecious lines are usually planted because the phenotype of gynodioecious plants is unstable under variable and extreme seasonality (Manshardt, 1999). Gynodioecious and androecious plants are mixed in the orchard with a ratio of 10 to 25 female plants to 1 male.

Gynodioecious lines are usually confined to tropical areas because flower development in hermaphrodite plants is highly sensitive to climatic stresses and the tropical climate can be more stable and benign for this tropical species. The hermaphrodite plants are susceptible to carpel abortion, or to transformation of stamens into carpel-like fleshy structures (carpellody) so ovarian development is variably expressed. Either female-type or deformed fruits are produced; both are unmarketable. In gynodioecious lines, all plants may be fully productive, as fruit is borne on both the hermaphrodite and the female (gynodioecious) plants. In some gynodioecious lines, the hermaphrodite plants frequently produce irregular fruits and their crop also is not as heavy as from the female plants (Shetty, 1953; Persley and Ploetz, 2003). However, in some gynodioecious lines the hermaphrodite fruits are preferred in some areas, and the female

plants are removed. For example, the hermaphrodite plants of the variety Solo produce pear-shaped fruits with more flesh and a smaller seed cavity, which are favored in Hawaii over the spherical fruits on female plants (Arkle and Nakasone, 1984).

## B. Sexual reproduction

The categories of unisexual and bisexual flower types occurring variously on plants have been described by many investigators, and have resulted in conflicting descriptions of plant sexual types (Sakai and Weller, 1999). The categories of the papaya sexual types from crosses are complex, but can be summarised simply; Table 1.23 shows the results of such crosses.

**Table 1.23 Formation of individual plant sexual types following papaya crosses (adapted from Storey, 1976)**

Flowers and plant: S = staminate (male); P = pistillate (female); H = hermaphrodite (male & female)				
Mating	S	P	H	(Non-viable zygote)
S × P	1	1	0	0
H × P	0	1	1	0
S × H	1	1	1	1
H × H	0	1	2	1
S × S*	2	1	0	1

\*Cross accomplished when sex reversal occurs on staminate (e.g. ambivalent male) plants.

The genetic or chromosomal mechanism for this complicated pattern of reproduction is not understood fully; a simple explanation has been used to represent the underlying condition. M is designated as a gene's dominant allele for maleness,  $M_H$  the dominant allele for hermaphroditism, and m the recessive allele for femaleness. Zygotes with homozygous dominant alleles (MM,  $MM_H$ ,  $M_HM_H$ ) are considered lethal; thus only Mm (male plants),  $M_Hm$  (hermaphrodite plants), and mm (female plants) are viable phenotypes (Hofmeyr, 1938a, 1938b, 1939a, Storey, 1938; Muthukrishnan and Irulappan 1985; Ma *et al.*, 2004). Storey (1953) furthermore proposed that tightly linked genes on a chromosome, determining sex, lethality, and additionally other sexual characteristics (e.g. inflorescence branching and number of flowers, petal fusion, stamen number, ovary shape), would be consistent with the crossing results. Hofmeyr (1967) hypothesised that M ( $M_1$ ) and  $M_H$  ( $M_2$ ) represent regions of slightly different length from which vital genes are missing. Sondur *et al.* (1996) accounted for the observations using recent knowledge about development of floral organs in other plants. They proposed that *trans*-acting regulatory proteins induce the sexual forms: an M allele of the sex locus (*Sex1*) induces male floral parts while inhibiting carpel development, an  $M_H$  allele induces male parts while only reducing carpel size, whereas the m allele has no ability to induce male parts. Lethality in the dominant homozygotes could result from loss of an essential function when the m allele is lacking. Sex reversal is rare in males, but its occurrence occasionally in hermaphrodites might result from the interaction between  $M_H$  and the gene's target (a promoter sequence or another protein factor) being less stable than the interaction between M and the target. Embryos from anther culture have produced only female plantlets (perhaps haploids or polyploids, e.g. dihaploids), which presumably originated from microspores, with only the m genotype involved because of the lethality related to the dominant alleles (Rimberia *et al.*, 2005).

A mutation recently discovered in a hermaphrodite Sunrise Solo cultivar produces exclusively hermaphrodite plants following self-pollination (i.e. no females in the expected 2:1 H:P ratio) (Chan-Tai *et al.*, 2003). Pollen of the mutant fertilising typical hermaphrodite plants produced a 3:1 ratio of hermaphrodites to females, indicating that all genotypes survived. Randomly self-pollinating these  $F_1$ s produced segregating  $F_2$ s that confirmed there were surviving homozygous dominant plants ( $M_HM_\oplus$ ) apparently with a new hermaphrodite allele (a variant of  $M_H$  designated as  $M_\oplus$ ). Furthermore, a new recessive lethality gene (l) was inferred that is linked to m, and lethal in female (mm) genotypes when homozygous recessive (mlml).

Based on interspecific hybridisation research in Caricaceae, Horovitz and Jiménez (1967) proposed an XX-XY system of sex determination. Micheletti de Zerpa (1980) studied the meiosis of their BC<sub>2</sub> of *Vasconellea cundinamaricensis* into *V. stipulata*, finding that 2 (only) of the 18 chromosomes had limited pairing and they sometimes behaved as univalents. This was taken as evidence that the Y chromosome of a *V. cundinamaricensis* with a bisexual phenotype had been transferred with its bisexuality genotype to produce the bisexual BC<sub>2</sub>, as *V. stipulata* is dioecious. (Both parental species showed no heteromorphic chromosomes.) Liu *et al.* (2004) found that the sex-determining genes in *Carica papaya* are located in a 4.4 Mb region of chromosome LG1 (linkage group 1), c. 10% of the chromosome, which shows suppressed recombination (Ma *et al.*, 2004). Thus LG1 acts like an incipient Y chromosome, in which a part is Y-like but the rest is autosome-like. This male-specific Y region (equating to c. 100-200 genes in average parts of the papaya genome) contains the non-female DNA coding, i.e. for male or hermaphrodite characteristics (Viskot and Hobza, 2004). The X-Y sequence divergence may be 10-20% (Charlesworth, 2004).

### C. Pollination

Cross-pollination may be common or infrequent, depending upon the papaya variety, flowering behavior (including flower type), and the environment. In some instances, male plants may more effectively pollinate hermaphrodites in adjacent orchards than the hermaphrodites can self pollinate. There may be genotype differences in variety lines, and seasonal changes in flower receptivity affecting pollination (Louw, 2000; A. Louw, 2003, Inst. Trop. & Subtrop. Crops, South Africa, pers. com.; Parés *et al.*, 2002; Parés-Martínez *et al.*, 2004). In gynodioecious plants, seed set was ten times greater when Coorg Honey Dew plants in India were hand-pollinated after being open-pollinated (Purohit, 1980), but hand-pollination did not increase papaya fruit set in Jamaica (Free, 1975). These results depend upon the pollinators available as well as the papaya variety; for example, bagging of hermaphrodite flowers of the variety Sunrise resulted in 90% fruit set, whereas for the variety Higgins it was only 33% (Rodríguez-Pastor *et al.*, 1990). In the dioecious condition the male and female plants are separate, so outcrossing is requisite to fertilisation.

In male flowers, stalked anthers (in an upper whorl) are exerted well beyond the floral tube's opening, whereas nearly sessile anthers (in a lower whorl) are inside the tube and dehisce (open) into it (Wiggins and Porter, 1971). In a dioecious variety (Washington), anther dehiscence was completed 36 to 18 hrs before flower opening (anthesis), and stigmas became receptive a day before anthesis (Khuspe and Ugale, 1977). In gynodioecious varieties, self-fertilisation is possible in hermaphrodite flowers. Anthers dehisce before anthesis, facilitating cleistogamy (Rodríguez Pastor *et al.*, 1990; Chan *et al.*, 1999; Ronse Decraene and Smets, 1999). The anthers of male, and functionally male (type 4+) flowers have been found to dehisce 2 days before anthesis, whereas the anthers of hermaphrodite flowers dehisce 1 day before anthesis (Parés *et al.*, 2002; Parés-Martínez *et al.*, 2004). Maximum stigma receptivity has been found to occur on the day of anthesis, although stigmas may become receptive 3 days earlier, and remain so for up to 5 days after anthesis (Subramanyam and Iyer, 1986; Dhaliwal and Gill, 1991). A hermaphrodite flower's pollen may be released before its stigmas' are receptive (protandrous dichogamy), with the stigmas becoming receptive only at anthesis (e.g. in the variety Cartagena Amarilla) (Parés *et al.*, 2002).

Pollen can be produced year-round. The grains are relative large (32-39 µm diam), and in the subtropics can be larger in local warmer areas (Sippel and Holtzhausen, 1992); the surface is finely reticulate (Allan, 1963a; Fisher, 1980). Viability of pollen (measured by stainability and germination) may vary seasonally, being highest in the rainy season and spring (Singh and Sharma, 1997), and much reduced in winter in subtropical locales such as Australia (Garrett, 1995, in OGTR, 2003a; Allan, 2002). Pollen may be relatively long-lived; in a Petri dish at room temperature, 16% of pollen grains remained viable for 16 days (Sharma and Bajpai, 1969; cf. Vahidy and Nafees, 1973).

Some pollen transport may occur by wind transfer, but the detection of pollen near plants seems to be meager (Allan, 1963c). Nontransgenic plants were grown *c.* 396 m (1300 ft) downwind of 0.4 ha of GUS-marked transgenic Rainbow papaya in Kapoho, Hawaii; no GUS expression was detected in the progeny of the nontransgenics (Manshardt, 2002, pers. com.). Purseglove (1968) noted that isolated female trees were pollinated as far as 244 m away from male trees, but Baker (1976) speculated that the observations might be explained by parthenocarp, which is known to occur in some varieties (*e.g.* Wettstein *et al.*, 1944; Free, 1975; Rodríguez Pastor *et al.*, 1990; Garrett, 1995, in OGTR 2003a). In a study designed to differentiate wind and insect importance, pollination was 38% in open-pollinated plants (Cera-type) but only 26% in controls that were muslin-bagged at the point of anthesis, thus suggesting a substantial contribution by insect pollinators at the suboptimum test site in Veracruz, Mexico (Mateos Sánchez *et al.*, 1995). After excluding medium and larger sized insects with wire gauze in South African papaya, no normal fruit set was observed (Allan, 1963c). In some areas, hand-pollination is infrequently undertaken to assist fruit set (Calif. Rare Fruit Growers, 1997).

The flowers open in the early night-time (Mekako and Nakasone, 1975a; Sippel *et al.*, 1989; Parés *et al.*, 2002), or the morning (Khuspe and Ugale, 1977; Azad and Rabbani, 2004), and since they are strongly dimorphic or polymorphic, provide different cues to potential insect pollinators. Staminate flowers may be more fragrant and open for 24 hrs, and they produce calcium oxalate crystals in the anthers and nectar basally (from the rudimental pistil), thus being an attractant for insects. The pistillate flower has no nectar, but a sweet non-sugar exudate seems available on its flared large antler-like stigmas (or stigmatic lobes) (Ronse Decraene and Smets, 1999; Parés *et al.*, 2002), and in these ways it may mimic the male flower (Baker, 1976). The female flowers may remain open for 7 days (Mabberley, 1998).

The main pollinators are somewhat unclear. Details of pollination especially by hawkmoths (Sphingidae), and apparently also mosquitoes, midges and thrips have been described (Heide, 1923; Free, 1975; Baker, 1976; Knudsen and Tollsten, 1993; Garrett, 1995, in OGTR 2003a; Morrisen *et al.*, 2003). In the Galápagos Islands, hawkmoths often visit the flowers after dark (McMullen, 1999); in mainland Ecuador, visitors to the male and female flowers include beetles, flies and mosquitoes (Nielsen, 1998, in Ronse Decraene and Smets, 1999). In Venezuela, 17 species were identified as pollinators (or visitors), including *Trigona* and *Xylocopa* bees (Marín Acosta, 1969). In Mexico (Veracruz), 712 insects in 68 families and 12 orders were recovered from 100 flowers, but only 38% pollination was attained (Mateos Sánchez *et al.*, 1995). In Central Amazonia (Brazil), papaya pollen is among the preferred foods of the bee *Trigona williana* almost year-round (Marques-Souza *et al.*, 1996). Similarly, honeybees were found transporting papaya pollen in South Africa (Allan, 1963c).

In some countries the role of insects in papaya pollination is factored as prominent, whereas in others wind-borne pollen appears to be more the concern. Accordingly, different recommendations for appropriate isolation distances from other papaya may reflect the specific conditions at different locations of production. Recognising both insects and wind as agents for pollen movement, Singh (1990) recommended 2-3 km isolation for production of foundation seed, but cited no experimental observations supporting this distance. The Hawaiian Identity Preservation Protocol for non-GMO papaya seed production specifies at least 1320 ft (400 m) isolation from other varieties (Hawaii Dept. Agric., 2003), based on the transgenic field test reported earlier in this subsection. The Papaya Biotechnology Network of Southeast Asia proposed that nontransgenic papaya should be separated by 400 m from any transgenic papaya plants that could bear anthers in field tests (Anon, 1999). USDA-APHIS approves an isolation distance of 500 m for papaya field tests in Florida. The Gene Technology Regulator (Australia) allows field testing only under conditions of complete insect exclusion by netting and removal of all male inflorescences (OGTR, 2003b).

#### D. Induced alterations to sexual development

Changes in environmental conditions (temperature, humidity, soil water, nitrogen) can induce various alterations in flowering and reproduction of papaya (Lange, 1961; Singh *et al.*, 1963; Rojas *et al.*, 1985; Terra de Almeida *et al.*, 2003a). When gynodioecious trees develop in hot and dry conditions, the inflorescence at each node may form a terminal bisexual flower but become subtended by male (staminate) axillary flowers (Manshardt, 2002, pers. com.). The male flowers can attain to 80% of all inflorescences on Solo cultivars during such conditions (Nakasone and Paull, 1998). When cooler conditions predominate, the axillary flowers may revert towards a preponderant bisexual morphology.

Sex reversals are also observed in the opposite direction, toward female structures. In both dioecious and gynodioecious lines, high temperatures and increased humidity can cause a shift towards female flowers (Singh, 1990). Lower night-time temperatures in the winter months in Hawaii may occasionally induce carpellody, in which stamens develop to resemble carpels, but associated with a developing fruit (Awada, 1958; Hsu, 1958a). The result is fruits of irregular shape, which are unmarketable (Chandrasekaran *et al.*, 1950; Watson, 1997). Carpellody may routinely reach 10-15% in hybrid and inbred lines in subtropical Australia (Grant, 2004).

In dioecious cultivars, where trees bear either male or female flowers, night-time temperatures below 12°C may induce the formation of bisexual flowers on male plants (Allan *et al.*, 1987), and short-day warm conditions may support this change (Aquilizan, 1987). Resistance to sex reversal of male trees is greater in spring compared to the cooler seasons (Allan *et al.*, 1987). In subtropical climates, fruit set on male trees (ambivalent males) may occur predictably (Watson, 1997). This conditional reversal is used to insure inbreeding of basically dioecious lines in Queensland (Aquilizan, 1987). In the tropics, the trauma of a few machete slashes to the trunk may stimulate male trees to produce bisexual flowers (Duke, 1967).

The female reproductive structures are stable, in contrast to the bisexual and male structures. Nonetheless, the fertility of female plants can be altered by environmental conditions. Low moisture levels or low nitrogen can induce female sterility (Awada and Ikeda, 1957).

### 6. Hybridisation

#### A. Interspecific crosses with *Carica papaya*

By the traditional methods of hybridisation, most attempts to transfer traits from *Vasconcellea* species into *C. papaya* have resulted in endosperm failure (Horovitz and Jiménez, 1958, 1967; Mekako and Nakasone, 1975b; Manshardt and Wenslaff, 1989a). Using embryo rescue and micropropagation techniques, some intergeneric hybrids have been generated (Manshardt and Wenslaff, 1989a, 1989b). Although F<sub>1</sub> plants from crosses with *C. papaya* were produced, they generally were sterile, and produced no F<sub>2</sub>s (Manshardt and Drew, 1998). Failure of meiosis resulted in formation of unreduced gametes, which in backcross to *C. papaya* have produced sterile sesquidiploid plants (Manshardt and Drew, 1998). In the Philippines, sterile F<sub>1</sub> hybrids of *C. papaya* with *V. cundinamarcensis* (synonym *V. pubescens*) and as well with *V. quercifolia*, *V. stipulata*, and *V. cauliflora* have been reported (Magdalita *et al.*, 1997b, 1998; Siar *et al.*, 1998; Villegas, 1999). Crossing various *C. papaya* with *V. cauliflora* in Venezuela achieved 0-76% fructification (Vegas *et al.*, 2003). All crosses using *V. cundinamarcensis* with *C. papaya* have produced infertile female hybrids (Drew *et al.*, 1998; R. Drew, 2001, Griffith, Univ., Australia, pers. com.).

Drew *et al.* (1998) achieved a limited fertile crossability of *C. papaya* with *V. quercifolia*. Large numbers of F<sub>2</sub>s were formed following embryo rescue, and backcrossed to *C. papaya*, which produced one male (BC<sub>1</sub>) that was fertile and tolerant to papaya ringspot virus; further development by backcrossing it was planned (Drew 2004, pers. com.). Sajise *et al.* (2004) have backcrossed an F<sub>1</sub> (from Drew) with elite

papaya lines in the Philippines and obtained 24 BC<sub>1</sub> plantlets. Also, some *C. papaya* crosses with *V. parviflora* have produced F<sub>1</sub> plants with viable pollen (Drew *et al.*, 1998).

Using RAPD techniques (with 14 primers), Jobin-Decor *et al.* (1997) estimated relatedness of some species of Caricaceae. *Carica papaya* had a mean dissimilarity of 69% with six *Vasconcellea* species; it had a dissimilarity of 84% with *Jacaratia spinosa* (and no isozymes in common). There were similar results for *C. papaya* with these *Vasconcellea* species using isozyme analysis — 70% dissimilarity. Using the amplified fragment length polymorphism (AFLP) technique (with 5 primer combinations to generate nearly 500 polymorphic bands), Van Droogenbroeck *et al.* (2002) grouped taxonomic accessions; cluster analysis revealed evidence for strong genetic divergence of *C. papaya* from all eight (plus unidentified) *Vasconcellea* species. In another AFLP study, Kim *et al.*, (2002) found that six *Vasconcellea* species were only 43% similar to *C. papaya*, but were 73% similar to one another. Using RFLP analysis of chloroplast and mitochondrial non-coding DNA, Van Droogenbroeck *et al.* (2004) found six *Vasconcellea* species to be more similar to *C. papaya* than to eleven other *Vasconcellea* species, which suggests further possibilities for interspecific crossing with papaya. These more closely related taxa include *V. quercifolia*, *V. weberbaueri* and *V. xheilbornii*, and less closely also *V. parviflora* and *V. stipulata*, but do not include *V. cundinamarcensis* or *V. cauliflora*.

## **B. Interspecific crosses within *Vasconcellea***

Workers hope to find a *Vasconcellea* bridge species for crossing with other *Vasconcellea* species, and so interbreeding with *Vasconcellea* known to cross with *C. papaya*. Natural hybrids between some species of *Vasconcellea* occur in the Andes (Badillo, 1971, 1993; Van Droogenbroeck *et al.*, 2004). The parentage of the sterile *V. xheilbornii* is uncertain, as molecular data (Jobin-Decor *et al.*, 1997; Aradhyia *et al.*, 1999; Van Droogenbroeck *et al.*, 2002, 2004) do not clearly support the usual interpretation that these wild and semi-domesticated plants (babaco, higacho) came from *V. cundinamarcensis* × *V. stipulata* (Badillo, 1967; NRC, 1989; Jiménez *et al.*, 1999). Organellar genome patterns identical with *V. weberbaueri* were found (Van Droogenbroeck *et al.*, 2004). *Vasconcellea stipulata* nevertheless has been well documented experimentally to produce fertile hybrids with *V. cundinamarcensis*. Similarly, when *V. stipulata* is the pollen parent in crossing with *V. xheilbornii*, progeny with 10–20 full seeds are produced (Horovitz and Jiménez, 1967; Micheletti de Zerpa, 1980). Sterile F<sub>1</sub> crosses have been made between *Carica papaya* and *V. xheilbornii* (as well as *C. papaya* and *V. stipulata*).

## **7. Genetic Variability**

### **A. Cytology and genome**

The diploid (2n) number of chromosomes of *C. papaya* is 18 (Meurman, 1925; Asana and Sutaria, 1929; Chen, 1993). No heteromorphic chromosomes have been detected (Datta, 1971; see Section V.B. Sexual reproduction), although differing chromosomal length and constriction morphologies have been found in various varieties. Tetraploids have been induced experimentally (Hofmeyr, 1945).

An analysis of the papaya nuclear genome has been undertaken by constructing a genetic linkage map (Sondur *et al.*, 1996). Using RAPD techniques for the analysis of a breeding line and a commercial line, evidence for 11 linkage groups was presented, and a total map distance of c. 1000 cM, compared to an expected genome size of c. 1350 cM. There was an overall low frequency of polymorphisms per primer (0.16) in comparison to other agronomic plants, suggesting either a relatively low genome size (including polymorphic repetitive DNA) in papaya, or low genetic diversity in the lines. The genome is small, with a 2C of 0.77 picograms and haploid DNA content of 372 Mbp (Arumuganathan and Earle, 1991). Making a map of the entire papaya genome is underway, with a constructed bacterial artificial chromosome (BAC) library of papaya that has nearly 40,000 clones (Ming *et al.*, 2001).

## B. Genetic variation within *Carica papaya*

The breadth of genetic variation readily available for papaya breeding and improvement is difficult to estimate. Papaya germplasm banks often hold a number of accessions, but the genetic resources in these repositories typically are modestly characterised. A typical collection contains a diverse assortment of *Carica* breeding material, cultivated types and cultivars, and often accessions of some *Vasconcellea* species as well. Surveys or analyses have been carried out to assess agronomic characteristics classically, and increasingly by molecular techniques (e.g. Santos *et al.*, 2003). Many agronomic descriptors for habit, flower, fruit, seed, etc., have been standardised by IBPGR (1988), including a range of alternative categories for the character. In a conventional analysis of a total of 125 accessions of the Solo group and the Formosa group (Tainung series) and a few intercrosses (holdings in one Brazilian repository), promising variability was found mainly in fruit size and tolerance to *Phytophthora* spp. (Dantas and Firmino de Lima, 2001). An AFLP analysis of 63 accessions from most growing areas (c. 17 countries) found an average similarity of 0.880 among them (Kim *et al.*, 2002).

### *Germplasm collections*

FAO's Seed and Plant Genetic Resources Service (AGPS) has a list of locations that cultivate papaya germplasm; these include nearly 90 research stations or seed production sites (FAO, 2001; cf. Bettencourt *et al.*, 1992). However, world germplasm resources for papaya are not organised in an accessible database. CIRAD-FLHOR (Centre de coopération internationale en recherche agronomique pour le développement) and IPGRI have a project for improvement of neotropical fruits which includes establishing a database with limited objectives. A regional effort for collecting and evaluating germplasm holdings of Caricaceae is being developed with a focus on resistance or tolerance traits, particularly for PRSV, bacterial decline, and anthracnoses — the regionally most important pathogens (Coppens, 2001, pers. com.). Other goals for Caricaceae germplasm collections may include development of *Vasconcellea* with potential as commercial fruits, and for new sources of enzymes such as papain (e.g. Colombo *et al.*, 1989; Villarreal *et al.*, 2003).

Breeding programs in various countries have established germplasm collections to co-ordinate with varietal improvement programs. The U.S. National Plant Germplasm System's USDA site in Hilo, Hawaii reports 153 accessions of *C. papaya* and several *Vasconcellea* spp. (GRIN, 2001); agronomic characters associated with specific accessions can be retrieved in a database via the Internet. Large holdings include those by Brazil (Coppens, 2001, pers. com.; Dantas and Firmino de Lima 2001) at EBDA-Bahia (82 accessions), EMBRAPA Mandioca e Fruticultura, Cruz das Almas, Bahia (141 accessions) and IAC-Campinas, São Paulo (169 accessions); Colombia at Univ. Nacional Medellín and CORPOICA (83 accessions) with additional accessions at other locations; India (90 *C. papaya* accessions) (Giacometti *et al.*, 1987); and Malaysia (72 accessions) (Chan *et al.*, 1999).

Most of the germplasm collections consist of living plants, but some include seeds (Giacometti *et al.*, 1987). Seed may be stored for up to 12 months at 12°C if capped in a tightly fitting jar, and longer under conditions specified by IBPGR (Giacometti *et al.*, 1987). Pollen likewise can maintain viability if stored appropriately, such as for 6 months either at 10°C and 10% relative humidity (Allan, 1963b) or at -18°C (Cohen *et al.*, 1989), or for 10-16 months cryogenically — even with several thawings and refreezings (Ganeshan, 1986).

### *Cultivated varieties as sources of genetic variability*

Many varieties of papaya are typically cultivated within a country, and each is often quite localised. A catalogue made in one of the high production areas of East Java, Indonesia recorded at least 24 such varieties (Baswarsati *et al.*, 1985, in Setyobudi and Purnomo, 1999). Some papaya varieties have found international acceptance and are grown extensively worldwide. One is Solo, from which other lines have

been selected (such as Sunrise Solo). Eksotika, bred from backcrossing Subang 6 with recurrent parent Sunrise Solo, is the flagship variety in Malaysia for export (Chan *et al.*, 1999).

To determine variability among papaya varieties and the degree of relatedness of some cultivars, Stiles *et al.* (1993) used RAPD molecular techniques (with 11 primers amplifying 102 distinct fragments). The comparison among 10 varieties from Malaysia, Mariana Islands, Hawaii and Florida showed their least relatedness was c. 70%, and the most closely related cultivars at c. 95%. The genetic similarities were generally those expected from knowing the region of origin and breeding history of the variety. Kim *et al.* (2002) analysed 186 AFLPs to estimate genetic diversity within 63 papaya accessions from many international sources; the genetic diversity was quite limited. The average similarity was 0.880, and in a single growing region, such as within Solo-type hermaphrodite cultivars in Hawaii, the average was 0.921. Analysis using isozymes is also possible — a total of 29 alleles have been found in 11 loci that segregate independently (Morshidi, 1998).

Surprisingly, Kim *et al.* (2002) found that the dioecious cultivars (which are open-pollinated) did not have more genetic variability than the hermaphrodite cultivars, which are thought to be mainly self-pollinated and so presumed to be less genetically variable. The literature on papaya breeding includes a considerable number of reviews (Singh, 1990), and no inbreeding depression has been detected (Hamilton, 1954). Indeed, after the initial selection for new traits, inbreeding by sib-mating for about four generations is often the practice to establish new varieties. Hybridisation was typically not used in developing new varieties (Storey, 1953), reinforcing the belief that inbreeding depression is not an important issue in cultivating papaya. Notwithstanding, in development hybridisation is increasing, as shown by Australian varieties and Malaysia's success with Eksotika II (see also Section III.B.1. Seed and B.3. Varietal selection).

#### *Genetic variability in resistance to pathogens*

Several research programs have looked for plants with the ability to develop tolerance (*i.e.* an ability to be infected but with limited effects) or resistance (lack of susceptibility to infection) to PRSV, one of the most devastating pathogens of papaya. Complete resistance is preferable, but tolerance is a useful option. The dioecious line Cariflora developed in Florida has shown a high level of tolerance (Conover *et al.*, 1986). Researchers in the Thailand Department of Agriculture have selected a PRSV-tolerant variety called Thapra 2; the plants may become infected, but have mild symptoms or remain symptom free. A second tolerant Thailand cultivar, Pakchong 1, was developed at Kasetsart University (Kositratana *et al.*, 1999). In the Philippines, the variety Sinta has exhibited high tolerance (Villegas *et al.*, 1996). In Malaysia, hybrids have been made of the popular variety Eksotika with Tainung No. 5, which has tolerance to PRSV, and the later generations have reasonable or high levels of field tolerance and are under continuing selection (Chan and Ong, 1996; Chan *et al.*, 1999; Chan, 2004; Chan, Y.K., pers. com.). See Section IV. Pests and pathogens for details about the results of these approaches. Since the development of transgenic varieties expressing a virus coat protein to confer resistance to PRSV, less effort has been expended to develop tolerant varieties; the genetically engineered resistance is providing more substantial benefits (Ferreira *et al.*, 2002).

#### *Molecular approaches for agronomic improvement*

Mutagenesis has augmented common breeding practices for improvement, resulting for example in a dwarf papaya with higher fruit yield per unit area (Ram and Majumdar, 1981). An RAPD-based genetic linkage map (Sondur *et al.*, 1996) has been used to locate and characterise genes affecting growth (height and stem diameter) and time of first flowering, by an analysis of quantitative trait loci (QTLs) in an F<sub>2</sub> papaya population derived from the cross of a gynodioecious, tall, late-flowering variety and a dioecious, semi-dwarf, early-flowering selection (Sondur *et al.*, 1995). Three QTLs affecting rate of height increase

and final height were detected, four QTLs affecting rate of stem diameter increase and final diameter, and two QTLs affecting node at first flowering. This is viewed as the lower limit of major QTLs for these traits. Five of the QTLs were on linkage group 1 (LG1) and one QTL each on LG3, LG4, LG5 and LG10, considered a non-random distribution. The height-influencing QTLs accounted for 64% of the phenotypic variance in height increase, the stem-influencing QTLs accounted for 52% of the variance in diameter increase, and the QTLs influencing node at first flowering accounted for c. 30% of the variance in node (in which the first flower-bearing node ranged from the 15th to the 36th). Variance due to environment was estimated to be 20% for height increase and 25% for increase in diameter.

Because practical methods for transforming papaya have been developed and the biotechnology is becoming well refined (Fitch *et al.*, 1990; Pinto *et al.*, 2002; Zhu *et al.*, 2004; Wall *et al.*, 2004), and transgenic commercial lines have been approved and available since 1998 (Cai *et al.*, 1999; Ying *et al.*, 1999; Gonsalves, 2000), papaya is a focus for improvement using genetic engineering approaches. Programs are using such tools to transform various varieties, for example co-ordinated under the Papaya Biotechnology Network of Southeast Asia (ISAAA, 2001a), with a current focus on delayed ripening characteristics and resistance to PRSV. Commercial interests have developed papaya with altered fruit ripening to allow extended marketing. Field testing in Australia has been authorised for papaya transformed with genes (*capacs1* and *capacs2*) that alter expression of ACC synthase, and with an ethylene expression gene (*ETR1*) (OGTR, 2003b). To increase tolerance to aluminum (common in tropical acidic soils), a transgenic papaya has been made in Mexico that overexpresses a citrate synthase gene from *Pseudomonas aeruginosa* (de la Fuente *et al.*, 1997). Transgenic papaya also is being researched as a delivery vehicle for an edible vaccine against tuberculosis (Zhang *et al.*, 2003).

Genes identified in papaya include some whose expression might be employed to modify various agronomic traits or enhance industrial production. Identified sequences (NCBI, 2001) include those affecting the following (Table 1.24):

**Table 1.24 Selected Papaya Genes for which Information is Available**

Industrial/Agronomic product	Carbohydrate metabolism	Others
a male-specific SCAR marker	sucrose synthase	arginine decarboxylase (ADC)
chymopapain	cell wall invertase	ATP synthase
papain	$\beta$ -galactosidase	membrane channel proteins
metallothionein-like protein	$\alpha$ -galactosidase	glutamine cyclotransferase
1-aminocyclopropane-1-carboxylic acid (ACC) synthase	xyloglucan endo-transglycosylase	caricain (proteinase omega)
		cysteine protease
		cysteine protease inhibitor
ethylene receptor	pectinesterase	Cu/Zn superoxide dismutase
		maturase K

Breeders and molecular biologists have a goal of developing resistance to various papaya diseases. Many pathogen-associated sequences have been cloned and identified (NCBI, 2001), which potentially could be employed in transformed papaya to provide endogenous resistance to pathogens that use papaya as host. Large numbers of viral genes have been sequenced, including coat proteins of numerous PRSV biotypes from different locations, a replicase, mRNA products of the virus, and an RNA polymerase gene (*NiB*). The whole PRSV and PapMV genomes have been sequenced. Other genes identified include two genes from PLDMV — an *NiB* gene and a coat protein (capsid protein) gene, a gene from the phytoplasma that causes papaya dieback (tuf) disease, the succinate dehydrogenase gene from the rickettsial bacteria that may cause papaya bunchy top disease; and an ileu tRNA.

Engineered resistance to viral diseases of papaya may require expression of geographically specific viral proteins. Many strains of the widespread PRSV may be virulent to papaya varieties even after they have been transformed with viral capsid sequences. For example some Florida (USA) isolates were

molecularly similar to Mexican and Australian isolates, but dissimilar to those from Asia (Davis and Ying, 1999). The genetically engineered resistance may be less or not effective if the origin of the capsid sequence is from a different region than the local viral strain (Tennant *et al.*, 1994, 2001, 2002). Recombination involving as few as 5 nucleotides in a virus coat protein gene can cause a susceptible response when a resistant papaya variety expressing the non-mutant coat protein is inoculated with the altered virus (Chiang *et al.*, 2000). However, not sequence similarity alone, but also gene dosage, plant stage, and other PRSV genes have important consequences for the expression of field resistance to PRSV (Tennant *et al.*, 2001; Tripathi *et al.*, 2004).

A consortium of scientists from universities, business and the U.S. Department of Agriculture (USDA-ARS) developed the first resistant papaya, which expressed a Hawaii-specific PRSV coat protein (Fitch *et al.*, 1992). Projects to deploy PRSV-resistant transgenic papayas are variously underway for example in Mexico, Guatemala, Jamaica, Venezuela, Brazil, Uganda, Tanzania, Bangladesh, Taiwan, Australia, and the countries that are members of the Papaya Biotechnology Network of Southeast Asia — Malaysia, Thailand, Vietnam, Philippines and Indonesia (Cai *et al.*, 1999; Flasiński *et al.*, 2002; Tennant *et al.*, 2002). Brazilian researchers working at Cornell University (USA) have transformed five papaya varieties using Brazil-specific PRSV capsid sequences, and planned field tests (ISAAA 2001b; Lima *et al.*, 2002). Thailand has developed a PRSV-resistant variety using a sequence from a PRSV strain specific to Southeast Asia; field tests were planned for 2002 (ISAAA, 2001c). Localised research efforts have succeeded in providing coat protein-based immunity in Australian and Venezuelan varieties (Lines *et al.*, 2002; Fermin *et al.*, 2004).

Several biotechnological solutions have been explored to supply the resistance needed to protect the crop from the prevalent PRSV of various regions. Chiang *et al.* (2001) suggested transforming papaya to express chimeric PRSV coat proteins, which possibly can be protective against these variable viral challenges. Bau *et al.* (2003) showed that a single coat protein sequence from a local Taiwan strain was adequate to provide complete immunity from heterologous strains arising in Mexico, Hawaii and Thailand, and this line did not produce any coat protein. Another approach may be to use the PRSV replicase gene to provide resistance (Chen *et al.*, 2001). An approach that produces an untranslatable product, which may result in an RNA-mediated immunity to PRSV, has been successful in protecting Australia cultivars (Lines *et al.*, 2002) and Florida cultivars (Davis and Ying, 2002). Also, programs for multiple protections against pathogens are attempting to combine coat proteins from PRSV with coat proteins from PLDMV (Maoka, 2002).

## 8. Ecology

### A. Dispersal

In Cameroon, forest elephants (*Loxodonta cyclotis*) seek papaya fruits beyond their protected reserve and disperse the seeds (Tchamba and Seme, 1993; Barlow, 2000). Wild *Carica papaya* seems to have many characteristics that fit the hypothesised megafaunal dispersal syndrome (Janzen and Martin, 1982; Barlow, 2000). The non-domesticated fruits are fairly large (5-8 cm in diameter) and visually nondescript (greenish unless fully ripened) but with a penetrating aroma, and are held high up on a trunk with suppressed branching. The fruits are indehiscent (without structural opening), and pulpy within but have peppery mustard-tasting seeds (Sharma and Singh, 1975; Passera and Spettoli, 1981) that are grouped centrally. Non-domesticated *Vasconcellea* fruits can be larger. Such unusual species may have evolved in response to consumption of fruits whole and seed dispersal by large (now extinct) mammals such as ground sloths (*Eremotherium*) and mastodon-like gomphotheres (*Cuvieronius*) (Simpson, 1969, 1980; Barlow, 2000).

A great many vertebrates with a wide variety of ecological roles eat papaya fruits and may disperse viable seeds. Coyotes (*Canis latrans*) in coastal western Mexico (Jalisco) habitually seek out papaya as food, sometimes taking fruits directly from the trees and causing important economic loss (Hidalgo-Mihart *et al.*, 2001). Pacas (*Agouti paca*), large forest rodents which range from Mexico to Paraguay, when in captivity selectively prefer papaya fruits because of their relatively high energy content (Laska *et al.*, 2003). Brow-ridged langur monkeys (*Trachypithecus* spp.) in eastern India (Tripura) raid the crop, and can cause tree mortality (Das, 1998). Arboreal neotropical monkeys consume papayas, such as wild cotton-top tamarins (*Saguinus oedipus*) and captive capuchins (*Cebus*) — which pass the seeds in less than 2 hrs (Wehncke *et al.*, 2003). Great fruit-eating bats (*Artibeus lituratus*), which are common from Mesoamerica and the Lesser Antilles to northern Argentina, disperse seeds found viable when tested (García *et al.*, 2000). In Papua New Guinea (Madang), papaya was found in fecal samples of lesser bare-backed fruit bats (*Dobsonia minor*) netted in the Kau Wildlife Area (Bonaccorso *et al.*, 2002).

In the Yucatán (southern Mexico), the plant is appropriately called papaya de pájaro (bird papaya). Many birds eat the fruits and may disperse seeds, such as Montezuma oropendolas (*Psarocolius montezuma*), a blackbird ranging from southern Mexico to central Panama (Webster, 1997), and Guianan cocks-of-the-rock (*Rupicola rupicola*) (Gilliard, 1962). Papaya was 29% of the diet of West Indian red-bellied woodpeckers (*Melanerpes superciliosus*) studied on Grand Cayman Island (Cruz and Johnston, 1984). Endangered Ouvéa parakeets (*Eunymphicus cornutus uvaensis*) of New Caledonia preferentially consume papaya which are available throughout the year in Melanesian gardens (Robinet *et al.*, 2003). Various bird species in India (Punjab) seek out the fruit (in rind-forming through ripening stages), and can result in crop damage of at least 3.4% (Mahli, 2001).

### B. Weediness

Papaya in different regions is variously described as an incidental escapee from cultivated sites, an opportunist, a pioneer species, or sometimes as an invasive or potentially invasive species. Papaya may persist beyond cultivation for indefinite periods. Little and Wadsworth (1964) state that “Through the tropics they grow almost as weeds, bearing fruit the first year from seed and spreading along roadsides and in waste places”; they report that in Puerto Rico papaya is widely cultivated, escaping, and naturalised. In the Galápagos Islands (Santa Cruz), papaya was found along a new road from the coast inland in the arid, transition, and humid zones, but did not persist along the old road (Haro Martínez, 1975). Papaya is usually not characterised as an invasive species (USDA-APHIS, 1997).

*Carica papaya* is regarded as a pioneer species in fairly natural habitats. Papaya can occur in forest gaps and within the early succession, since it has such characteristics as rapid growth in response to disturbance and high light intensity, and prolific production of seeds and an attractive fruit. Its pioneering ecological strategy includes a short life cycle with seed dormancy and a seed bank.

As an opportunist, papaya has the capacity to establish significant seed banks. In Central Amazonia, Brazil (Santarém region) at some old Dark Earth locations (aboriginally cultivated soils), papaya comes up after the long-standing tropical rain forest has been cleared and burned (Clement *et al.*, 2004). In a post-hurricane study of regeneration that compared feral *C. papaya* with a similar-sized native pioneer tree species (*Trema micrantha*) in Florida hammock habitats, papaya had a broader niche for regeneration (Kwit *et al.*, 2000). They averred that dormant seed supply seed for population return following natural disturbance. Moist wild papaya seeds kept in total darkness in Petri dishes at room temperature remain viable and dormant (Vázquez-Yanes and Orozco-Segovia, 1996; cf. Pérez-Nasser and Vázquez-Yanes, 1989). In Japan’s Bonin Islands (Hahajima) in mesic subtropical forest, the seed bank at 16 sites was sampled (0–20 cm) in three layers. Viable papaya seeds occurred in all three, with the most seeds at a depth of 4–10 cm, where their density was c. 18 per m<sup>2</sup> (Yamashita *et al.*, 2003).

Nakasone and Paull (1998) characterise papaya as “a rapid volunteer in areas where the tree vegetation has been disturbed”. Subsequent to major hurricane damage (1992) in southern Florida (USA), papaya recruited abundantly and rapidly in unmanaged and managed areas. In the 1st and 2nd years, it comprised 76% and 40% of all stems respectively in the unmanaged areas (Horvitz and Koop, 2001). In an inventoried natural semi-evergreen forest of southeastern Mexico on the Yucatán Peninsula (Quintana Roo) where no papaya had been recorded, papayas were infrequently present just 5 months after an extreme hurricane had altered the region (Sánchez-Sánchez and Islebe, 1999).

Randall (2002) reported weedy papaya infestations on some tropical islands and in localised areas of New Zealand. On Christmas Island (Indian Ocean) and in the Mariana Islands (Rota) and Samoa (Savaif), papaya is a colonising invader of disturbed or burned habitats (Craig, 1993; Space *et al.*, 2000; Elmqvist *et al.*, 2001; Green *et al.*, 2004). On Tongatapu, papaya was found in 44% of 52 sample plots in a range of land-cover types (especially fragmented interior forest). Following from these observations, Wiser *et al.* (2002) stated that it should be considered a potentially serious invader. In coastal Queensland (Australia), “small, low-density self-perpetuating populations” may be found (OGTR, 2003a). In the Hawaiian Islands, papaya is sparingly naturalised on four main islands, with some plants even occurring on nearly vertical rock faces (Wagner *et al.*, 1999; Oppenheimer and Bartlett, 2000). In a wet-forest region of coastal Ecuador near the Andean foothills, Dodson and Gentry (1978) found papaya to be common in second-growth areas, including a forest regenerating from an agricultural clearing about 18 years previously.

Papayas are sensitive to most herbicides and volunteer plants in agricultural habitats can be eliminated using paraquat, glyphosate, or triclopyr (Lee, 1989; Kline and Duquesnel, 1996). Reports are scarce on efforts to reduce feral *C. papaya* in relatively natural habitats (e.g. Horvitz and Koop 2001), which may be important for ecological restoration or to reduce genetic contamination in orchards from feral off-types.

### C. Optimal habitats

*Carica papaya* requires a tropical or semitropical habitat that is always rather warm and provides high illumination. Depending on latitude, cultivated varieties may thrive at elevations from sea level to 600 m and may range up to about 1200 m, being limited by the occurrence of killing frost (Arntzen and Ritter 1994; Bhattarai *et al.*, 2004). Temperatures below 11°C negatively affect growth and fruit set, and strongly retard fruit maturing and ripening (Shetty, 1953; Allan 2002). At higher elevations fruit tends to be insipid.

Rainfall must attain to at least 350 mm and should not exceed about 2500 mm, as excessive moisture is detrimental to the plant or fruit (Singh 1990). Within its probable native range in Veracruz (central-eastern Mexico) (Moreno, 1980), a study correlated regional parameters with 62 botanical collections (including 25% from scarcely or slightly modified habitats; cf. Del Angel-Pérez and Mendoza-Briseno 2004). Most plants were in Köppen's Am(f) subclimate type; the annual estimated total precipitation was 1200-1400 mm, with 100-150 days having considerable rain (an average 30-40 mm possible in 24 hrs), and with 20-40 days per year having essentially no rain (Gómez-C. 2000). Relative humidity of more than 60% may be optimal for papaya (FAO 1986); nevertheless, in South Africa the best-quality fruits are grown in low humidity regions (Malan 1953).

Acceptable growth of papaya can occur in a variety of soils. An optimal soil that promotes growth is well drained, and flooding is not tolerated (Malaysia Dept. Agric. 2001). A pH of 5.0-7.0 is favorable for its cultivation (Nakasone and Paull, 1998); uniform, rich loams of pH 6.5-7.0 are considered optimal (Singh, 1990). For Hawaiian soils, Younge and Plucknett (1981) recommended an optimum pH of 5.8-6.2; if the pH reaches 6.2-6.5, increased damage by *Phytophthora* may occur (Adlan, 1969). Arbuscular mycorrhizal fungi are strongly beneficial in cultivation (Jaizme-Vega and Azcón 1995; Vierheilig *et al.*, 2000; Trindade *et al.*, 2001). Compacted soils that impede root penetration (Yamanishi *et al.*, 1998) will also limit net CO<sub>2</sub> assimilation (Campostrini and Yamanishi 2001b).

Optimal growth is in sheltered locations; strong winds in combination with rain or low temperatures can cause fruit loss as a consequence of lodging that can easily occur in these fairly shallow-rooted plants. Most roots occur in the soil's upper 20 cm and they may extend radially to 80 cm (Fisher and Mueller 1983; Masri 1993; Malaysia Dept. Agric. 2001). Under optimal conditions taproots may reach a depth of about 1 m, and papaya rooting can adapt to slopes (Marler and Discekici 1997). With minimal pre-plant preparation or an absence of fertiliser applications, only a fibrous root system may develop (Younge and Plucknett 1981).

#### **D. Optimal geographic location**

The genetic diversity within the present commercial cultivars provides relatively wide adaptability for papaya and permits cultivation in many locations. Production of this world crop is generally found between 30°N and 40°S, but commercial production is compressed to a circumferential region around the equator from 25°N to 25°S (Singh 1990). When grown outside these tropical latitudes, optimal growth is in well-protected areas near sea level (Nakasone and Paull 1998). Photosynthetic saturation occurs at rather high irradiance, and shade induces major morphological and cellular changes (Imai *et al.*, 1982; Buisson and Lee 1993; Marler 1994); papaya has been described as a shade-avoiding species (Grime 1981). Papaya is cold-sensitive, wind-sensitive, flooding-intolerant, and moderately salt-sensitive (Marler 1994; Clemente and Marler 2001). Nevertheless, it has been successfully adapted as a dooryard treelet, and has naturalised in many locations. On most continents and on many islands, the usefulness of papaya's products and its wide range of traits have allowed the papaya a place in many gardens, local markets and commercial enterprises.

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## SECTION 11 OYSTER MUSHROOM (*PLEUROTUS* SPP.)

### 1. General Information

Oyster mushroom is regarded as one of the commercially important edible mushrooms throughout the world. It consists of a number of different species including *Pleurotus ostreatus*, *Pleurotus sajor-caju*, *Pleurotus cystidiosus*, *Pleurotus cornucopiae*, *Pleurotus pulmonarius*, *Pleurotus tuber-regium*, *Pleurotus citrinopileatus* and *Pleurotus flabellatus*. They thrive on most of all hardwoods, wood by-products such as sawdust, paper, pulp sludge, all the cereal straws, corn and corn cobs, coffee residues such as coffee grounds, hulls, stalks, and leaves, banana fronds, and waste cotton often enclosed by plastic bags and bottles. The oyster mushroom is the second most important mushroom in production in the world, accounting for 25% of total world production of cultivated mushrooms. Oyster mushroom is grown world-wide, and China is the major producer. *P. ostreatus* was first cultivated in the USA in 1900 and several other species of the oyster mushroom such as *Pleurotus sajor-caju* were initially cultivated in India after the late of 1940s. The oyster mushroom has been regarded as one of the most profitable cash crops in Korea, accounting for 65% of total domestic mushroom production.

This consensus document which describes the main aspects of the biology of Oyster Mushroom was prepared by the lead country, Korea, to provide background information for science-based decision making in consideration of future release of transgenic mushrooms into the environment. Included are description of the taxonomy and natural habitat of the genus *Pleurotus* and morphological description of *Pleurotus ostreatus*, the agronomic practices, the life cycle and sexual reproduction, and genetics. *Pleurotus ostreatus* is the main focus of this document, but other species of the oyster mushroom are also covered in this consensus document.

### 2. Taxonomy and Natural Distribution

#### A. Taxonomy and nomenclature

Oyster mushroom, *Pleurotus* spp., belonging to the genus *Pleurotus* (Quel.) Fr., tribe Lentineae Fayod, family Polyporaceae (Fr.) Fr., is widely distributed throughout the Northern Hemisphere, such as Europe, North Africa, Asia and North America (Singer, 1986). To date, approximately as many as 70 species of *Pleurotus* have been recorded and new species are discovered more or less frequently although some of these are considered identical to previously recognised species. The genus *Pleurotus*, which was first recommended as a tribe within genus *Agaricus* by Fries (1821), was proposed as a genus by Quelet (1886). Three genera of this group, *Pleurotus*, *Lentinus*, and *Panus*, were possible to be separated according to their anatomic characters of the sterile tissues of the hymenophores as being homogeneous taxonomic groups. Hilber (1982) recommended that crossing of monospore cultures is a valuable basis for *Pleurotus* studies. *Pleurotus ostreatus* (Jacq: Fr.) Kummer is the most cultivated species among the oyster mushroom and the type species of the genus *Pleurotus*.

Recently, the majority of mycologists have followed the proposition made by Singer (1986) which divides the genus *Pleurotus* into six sections: Sect. *Lepiotarii* (Fr.) Pilat, Sect. *Calyptrati* Sing., Sect.

*Pleurotus* Sing., Sect. *Coremiopleurotus* (Hilber), Sect. *Lentodiellum* (Murr.) Sing. and Sect. *Tuberegium* Sing.. *Pleurotus ostreatus* was placed in the Sect. *Pleurotus* based on the absence of veil and with the monomitic hyphal system.

## B. Morphological description

Species identification within the genus *Pleurotus* is difficult because of the morphological similarities and possible environmental effects. Mating compatibility studies have demonstrated the existence of eleven discrete intersterility groups in *Pleurotus* to distinguish one species from the others. *P. columbinus*, *P. florida*, *P. salignus*, and *P. spodoleucus* are the synonyms or subspecies taxa for the species of *P. ostreatus*.

### *Macroscopic features of Pleurotus ostreatus* (Jacq.: Fr.) Kummer

- Pileus: 40-250mm broad, oyster-shape, spatulate to lingulate when young, convex then later becoming conchate to flabellate, surface smooth, grey lilac, violet-brown to lilac blackish when young later becoming cream-beige, but usually very variable in colour, margin smooth when young, later somewhat undulating and striate. For descriptions of macroscopic features of fruiting bodies, descriptions and illustrations of microscopic characters, and distribution of this taxa, references of Breitenbach and Kranzlin (1991), Donk (1962), Imazeki and Hongo (1987), and Moser (1983) were referred to respectively. Colour names were taken from Kornerup & Wanscher (1983).
- Context: white to grey-white, thin to thick, fleshy, radially fibrous, odour fungoid, taste mild.
- Lamellae: long-decurrent, crowded, whitish to cream or pale greyish, edge smooth, later somewhat undulating, lamellulae 1- or 3-tiers.
- Stipe: 10-20×10-25mm, rudimentary, usually lateral, severa conrescent, surface longitudinally striate, whitish villose-pilose, context solid.

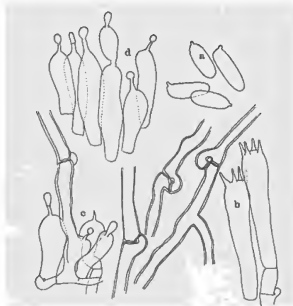
**Figure 1.5** Macroscopic feature of *P. ostreatus*



*Microscopic features of Pleurotus ostreatus* (Jacq.: Fr.) Kummer

- Spores:  $6.5\text{--}9 \times 2.8\text{--}3.5\mu\text{m}$ , cylindric to cylindric-ellipsoidal, smooth, hyaline, with vacuoles.
- Spore print dingy grey or pale lilac grey.
- Basidia:  $23.6\text{--}27 \times 5\text{--}7.5\mu\text{m}$ , slenderly clavate with 4-spored and a basal clamp connection.
- Hymenophoral trama: regular to irregular, trama monomitic.
- Cystidia: absent or cystioid, rarely seen.
- Pileipellis: composed of irregular, densely interwoven, flexuous and branched hyphae, usually  $2\text{--}4\mu\text{m}$  across, with brown pigment, somewhat gelatinised, septa with clamp connections.
- Habit & Habitat :Usually gregarious, clustered on the dead hardwood in park and both side of road, rarely on conifers, Suwon, Pochon, Cholwon, Whasong in Kyunggi Province , Gyeryong-san, Chilgap-san in Chungnam Province, Chiak-san in Kangwon Province, Kangjin in Chonnam Province and Hanla-san in Jeju Province in Korea. Spring to autumn.
- Distribution: Europe, America, North Africa, and Asia

**Figure 1.6** Microscopic feature of *P. ostreatus* (a: spores, b: basidia, c. cheilocystidia, d: pleurocystidia)



### C. Natural habitat

The geographic distribution of the oyster mushroom varies according to its species. For example, *P. pulmonarius* and *P. cystidiosus* are known to be distributed in the tropical and subtropical region, while *P. eryngii* are found in southern Europe, North Africa and central Asia. It has many subspecies and similar taxa such as *P. fuscus* var. *ferulae* from China. *P. ostreatus* is widespread in the temperate zones such as Korea and Japan because it forms fruit-bodies at relatively low temperature compared to other *Pleurotus* species. The geographic distribution of *P. tuber-regium* includes most of equatorial Africa, India, Sri Lanka, Southeast Asia, North Australia, and the southern Pacific countries as well (Table 1.25).

Commonly grown on broad-leaf hardwoods in the spring and fall, especially cottonwoods, oaks, alders, maples, aspens, ash, beech, birch, elm, willows and poplars are favoured natural habitat for oyster mushroom. Although seen on dying trees, *P. ostreatus* is thought to be primarily a saprophyte, but behaves as a facultative parasite at the earliest opportunity. Occasionally, it grows on composting bales of straw and in Mexico, on the pulp residues from coffee production. The most abundant fruiting of this species is in low valley riparian habitats (Stamets, 1993).

Table 1.25 Classification of the genus *Pleurotus* and its geographical distribution (Singer, 1986)

Sect.	Species	Geographical Distribution
Lepiotarii	<i>P.dryinus</i> (Pers: Fr.) Kummer	Japan, USA, Swiss, Germany, Sri Lanka, Portugal
	<i>P.dryinus</i> (Pers: Fr.) Kummer var. <i>tephrotrichus</i> (Fr: Secr.) Gill.	
	<i>P.rickii</i> Bres.	
	<i>P.lindquistii</i> Sing.	
Calyptrati	<i>P.calyptratus</i> (Lindb.) Sacc.	China
Pleurotus	<i>P.ostreatus</i> (Jacqu: Fr.) Kummer	Korea, China, Japan, USA, UK, Switzerland, Netherlands, Germany, Sri-Lanka, Portugal, Slovakia
	<i>P.ostreatus</i> (Jacqu: Fr.) Kummer var. <i>columbinus</i> (Quel. Apud Bres.) Quel.	Japan, USA, Germany, Slovakia
<i>Pleurotus columbinus</i> Quel.	<i>P.pulmonarius</i> (Fr.) Quel. : Fr.	Korea, China, Japan, Germany, Portugal, New Zealand
	<i>P.citrinopileatus</i> Sing.	Korea, China, Japan
	<i>P.ostreatoroseus</i> Sing.	
	<i>P.opuntiae</i> (Dur. & Lev.) Sacc.	
	<i>P.macropus</i> Bagl.	
	<i>P.laciniatocrenatus</i> (Speg.) Speg.	
	<i>P.euosmus</i> (Berk.) Sacc.	
	<i>P.phellodendri</i> (Sing.) Sing.	
	<i>P.araucariicola</i> Sing.	
	<i>P.pantoleucus</i> (Fr.) Sacc.	
	<i>P.prometheus</i> (Berk. & Curt.) Sacc.	
	<i>P.yuccae</i> Maire	
	<i>P.convivarum</i> Dunal & Delile	
	<i>P.parthenopejus</i> (Comes) Sacc.	
	<i>P.salignus</i> (Schrad.) Quel.	
	<i>P.importatus</i> Henn.	
	<i>P.gemmellari</i> (Inz.) Sacc.	
Coremiopleurotus	<i>Pleurotus cystidiosus</i> O.K. Miller	
	<i>P.abalonus</i> Han, Chen & Cheng	
Lentodiellum	<i>Panus concavus</i> Berk. <i>Pleurotus concavus</i> (Berk.) Sing.	China, Japan
	<i>P.levis</i> (Berk. & Curt.) Sing.	
	<i>P.strigosus</i> (Berk. & Curt.) Sing.	
	<i>P.fockeii</i> (Miquel) Sing.	
	<i>P.calyx</i> (Speg.) Sing.	UK
	<i>P.sajor-caju</i> (Fr.) Sing.	China, USA, Sri-Lanka, Australia
	<i>P.squarulosus</i> (Mont.) Sing. Ex Pegler	USA, Sri-Lanka
	<i>P.floridanus</i> Sing.	UK
	<i>P.subtilis</i> (Berk.) Sing.	
Tuberregium	<i>P.tuber-regium</i> (Rumph.Fr.) Sing.	China, Sri-Lanka, Australia

### 3. Agronomic Practices

*Pleurotus* spp. is generally referred as the oyster mushroom because the pileus or cap is shell-like, spatulate and the stipe is eccentric or lateral. *Pleurotus ostreatus* (Jacq.: Fr.) Kummer is one of the best known species among the oyster mushrooms. Other commonly cultivated species include *P. sajor-caju*, *P. cystidiosus*, *P. eryngii* and *P. tuberregium* (Chang and Miles, 1989). Various species of these wood-rotting fungi are found all over the world and this mushroom is especially appreciated in Asia for its edibility.

The oyster mushroom has many advantages as a cultivated mushroom: rapid mycelial growth, high ability for saprophytic colonisation, simple and inexpensive cultivation techniques and several kinds of species available for cultivation under different climatic conditions. In addition, oyster mushroom is low in calories, sodium, fat and cholesterol, while rich in protein, carbohydrate, fibre, vitamins and minerals. These nutritional properties make this mushroom as a very good dietary food. In addition, consumption of oyster mushroom has positive effects on the general human health because of a number of special substances (Kues and Liu, 2000). Owing to these attributes during recent years, the production and consumption of this mushroom has increased tremendously and is ranked second to the button mushroom. The high ability to degrade the lignin-cellulose of *Pleurotus* spp. was also used in eliminating of the xenobiotic pollutants such as pentachlorophenol (PCP), dioxin, polycyclic aromatic hydrocarbons (PAHs). This suggests the possibility of new usage of this mushroom for environmental bioremediation (Kubatova *et al.*, 2001; Hirano *et al.*, 2000).

Despite its usefulness as food and bioconversion materials, three notable disadvantages persist in the cultivation of oyster mushroom. First, the oyster mushroom is quick to spoil and so is presentable to the market for only a few days. Secondly, the spore load generated within the growing room can become a potential health hazard to workers thus pickers can become allergic to the spore. Sporeless strains, which tend to have short gills and are thicker fleshed, prolonging storage, are highly sought after by oyster mushroom growers. Thirdly, the growers must wage a constant battle against the intrusion of mushroom flies. Oyster mushroom attracts Sciarid and Phorid flies to a far greater degree than any other group of mushrooms.

**Table 1.26 Production of oyster mushrooms under commercial cultivation in some countries (Chang, 1993; Kues and Liu, 2000)**

(Unit: Mt)

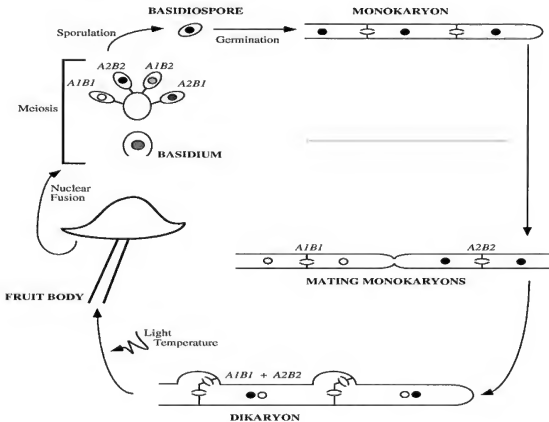
Oyster mushroom production			
Countries	1991	1994	1998
China	800 000	654 000	
Japan	33 475	20 441	
USA	695	900	
Indonesia	15 000	1 000	
Thailand	7 000	15 000	
Spain		100	
Netherlands		150	
Italy		1 500	
UK		150	
Germany		1 000	
France		2 000	
South Korea	51 782	72 810	75 684
Taiwan	3 500		
India	600		
Hungary	2 500		
Total	914 552	696 241	

#### 4. Life Cycle and Growth

##### A. Life cycle of *Pleurotus ostreatus*

The major events in the life cycle of *P. ostreatus* could be described as follows (Figure 1.27). A single basidiospore germinates to be a mass of homokaryotic mycelium, each cell of which contains a single haploid nucleus. The homokaryotic mycelia continue to grow until the hyphae fuse with the other hyphae which have compatible mating type. After fusion between compatible homokaryotic hyphae, reciprocal nuclear migration occurs and a heterokaryotic mycelium is formed. The subsequent growth involves the synchronous division of the two nuclei in each compartment and their regular distribution as nuclear pair throughout the mycelium via clamp connections. Heterokaryotised mycelia with enough mycelia mass and appropriate environmental stimuli (cooling 10 - 21°C, relative humidity 85-90%, and light requirement 1000-2000lux, CO<sub>2</sub> < 1000 ppm) can form the fruit bodies. During fruit body formation, nuclear fusion and meiosis occur only in the specialised basidia. Haploid nuclei migrate into a tetrad of basidiospores, external to the basidium. Each basidium has commonly four monokaryotic basidiospores. Occasionally five or more have been observed. These spores germinate into homokaryotic hyphae

Figure 1.7 Life cycle of the *Pleurotus ostreatus*



Source : Casselton, 1995

##### B. Requirement for mycelial growth

The carbon sources suitable for mycelial growth are starch, glucose, fructose, maltose, mannose, sucrose, pectin, cellulose and lignin. Ethanol is also a source of carbon for mycelial growth; however, citrate, oxalate and other organic acids are not beneficial to the growth of the mycelium. The nitrogenous

sources utilised by *Pleurotus* spp. are peptone, corn steep liquor, soybean cake powder, yeast powder, ammonium sulfate, asparagine, serine, alanine and glycine. The utilisation of urea is rather poor.

The optimal temperatures for growth of the mycelium are around 25-28 °C and the range of pH is about 5.5 to 6.5. The tolerance of mycelia for CO<sub>2</sub> is rather strong. The mycelia of *Pleurotus* spp. can still grow flourishingly at the carbon dioxide concentration of 15 to 20%. Only when the concentration of CO<sub>2</sub> is raised to 30% does the growth of mycelia rapidly decrease (Chang and Miles, 1989).

### C Requirement for fruit body formation

For fruiting body formation, CO<sub>2</sub>, light and temperature is key environmental factors. When the CO<sub>2</sub> concentration in the mushroom house or growing bags is higher than 600 ppm (0.06%), the stipe elongates and the growth of the caps will be prevented. The requirements for light are different for the various stages of growth. The growth of mycelium does not need any light and cultivation of the oyster mushroom in a dark place is better than in a bright place. The formation of primordia and the growth of fruiting bodies require light. The former requires light of 200 lux intensity for over 12 hrs. The growth of the fruiting body requires light of 50 to 500 lux intensity. The colour of the caps is closely related to the intensity of light, and if it is low, then the colour will be pale. The optimal temperatures for the development of fruiting bodies can range from 10 to 18 °C (Chang and Miles, 1989). Growers can choose a suitable strain for their own natural environment. Each *Pleurotus* species needs different environmental conditions for fruitbody development as illustrated in Table 1.27 (Stamets, 1993; Kang, 2004).

**Table 1.27 Environmental parameters for fruiting of oyster mushroom**

Species	Temp. (°C)	Relative humidity (%)	CO <sub>2</sub> (ppm)	Light (lux)
<i>P. pulmonarius</i>	21-29	90-95	<1,000	500-1,000
<i>P. cystidiosus</i>	21-27	85-90	<2,000	500-1,000
<i>P. djamar</i>	20-30	85-90	500-1,500	750-1,500
<i>P. eryngii</i>	15-21	85-90	<2,000	500-1,000
<i>P. euosmus</i>	21-27	90-95	<1,000	750-1,500
<i>P. ostreatus</i>	10-21	85-90	<1,000	1,000-1,500
<i>P. pulmonarius</i>	18-24	85-90	400-800	1,000-1,500
<i>P. tuberregium</i>	30-35	85-90	<2,000	

## 5. Sexual Reproduction and Crosses

### A. Mating system and gene flow potential

*P. ostreatus* is heterothallic (self-sterile) and sexual reproduction is governed by the mating type genes. Mating type genes prevent mating between genetically identical cells. *P. ostreatus* has a bifactorial tetrapolar incompatibility mating systems which has two unlinked mating type factors designated A and B (Eugenio and Anderson, 1968). Factor A controls nuclear pairing, clamp cell formation, coordinate cell division and clamp cell septation whereas factor B is responsible for the control of nuclear migration, septa dissolution and clamp cell fusion. Two monokaryotic mycelia are compatible if they have different alleles at both loci. Multiple allelism for mating type genes was first noted by Terakawa (1957) and amply demonstrated in a sample of over 20 dikaryons collected from nature by Eugenio and Anderson (1968). The latter investigators estimated that there are a total number of 63 A types and 190 B types in the natural world-wide population of this species. Because of this multiple allelism of mating type, the out breeding potential is estimated close to 100% in nature and the inbreeding potential can be as low as 25%.

The spore of *P. ostreatus* usually gets off the gill and away from the mushroom cap. Once the spores have cleared the bottom of the cap, air currents carry them away. When the spores are a few millimetres

away from the cap they can be picked up by the faster winds and carried considerable distances thus enabling them to cross with the same species. However, no data are available regarding how far they can travel into the open air. Due to its nature of heterothallism, the spores of *P. ostreatus* behave like open pollinated crops. Therefore, appropriate measures should be taken to avoid unwanted gene flow when *P. ostreatus* is cultivated.

### B. Interspecific cross

Interspecific cross was reported among *P. ostreatus*, *P. florida*, *P. columbinus* and *P. sapidus* (Peberdy *et al.*, 1993). These species are ambiguous in specification of *Pleurotus*. Some scientists said that the species are the same species. There are several reports concerning interspecific crosses involving *Pleurotus* species based on protoplast fusion (Yoo and Cha, 1993).

### C. Monokaryotic fruiting

Monokaryotic fruiting has been reported on more than 34 species in basidiomycetes (Stahl and Esser, 1976). *P. ostreatus* has also been found the monokaryotic fruiting (Kim, 2000). Esser *et al.* (1979) proposed that two genes, *fil+* and *fi2+*, are responsible for initiation of fruiting, and Kim (2000) demonstrated the mating type switching in the homokaryotic fruiting strains.

## 6. Genetics of *P. Ostreatus*

### A. Genome size

The study of genome organisation in *P. ostreatus* has been hampered by the small size of fungal chromosomes. Different authors reported different chromosome numbers and genome sizes for this species (Sagawa and Nagata, 1992; Peberdy *et al.*, 1993; Chiu, 1996). Recently, by using Pulse Field Gel Electrophoresis and linkage mapping, eleven chromosomes were resolved per haploid genome which added up to a total genomic size of 35Mb in average as shown in Table 1.28. Each chromosome has size from 1.4Mb to 4.7Mb. The use of chromosome-specific single-copy probes resolved the ambiguities caused by chromosome co-migration (Laraya *et al.*, 2000).

**Table 1.28 Estimated chromosome size of *Pleurotus* spp**

Chromosome	<i>P.ostreatus</i>	<i>P.florida</i>	<i>P.sajor-caju</i>	<i>P.pulmonarius</i>	<i>P.columbinus</i>	<i>P.sapidus</i>
I	4.70	5.1	5.70	5.70	5.70	5.50
II	4.35	4.7	5.10	5.30	4.70	4.60
III	4.55	4.1	3.10	5.10	4.30	4.30
IV	3.55	3.8	2.50	4.50	3.60	3.80
V	3.45	2.7	2.00	3.10	3.10	3.30
VI	3.10	2.2	1.60	2.70	2.50	2.30
VII	3.15	1.6	-	2.00	1.80	1.40
VIII	2.95	1.1	-	1.60	1.40	0.90
IX	2.10	0.7	-	-	-	-
X	1.75	-	-	-	-	-
XI	1.45	-	-	-	-	-
<b>Total genome size (Mb)</b>	<b>35.1</b>	<b>26.00</b>	<b>20.00</b>	<b>30.00</b>	<b>27.10</b>	<b>26.10</b>

Source : Peberdy *et al.*, 1993]

## **B. Linkage map**

Using 80 monokaryons derived from one commercial strain, segregation of 196 markers was studied. The linkage analysis allowed to associate the markers into 11 linkage groups which span a total of 1000.7 cM. Also this linkage map was used for QTL mapping associated with growth rate of monokaryon and dikaryon (Larraya *et al.*, 2000).

## **C. Transformation**

Although commercial transgenic mushroom strains are not available, molecular breeding studies of the mushrooms have been carried out world-wide. The Netherlands, the United Kingdom, Japan, Spain and the United States are among the leading countries in mushroom biotechnology including the development of transformation systems. Possible target genes for transformation include: senescence genes to improve mushroom quality; substrate utilisation genes to enhance yields; and developmental genes to control mushroom fruiting. There are numerous potential pest and disease resistance targets, including genes involved in response to fungal pathogens, toxicity to insects and natural pest resistance. In addition, transformations with mating type genes that regulate inter-strain compatibility can alter breeding behaviour.

Transformation of *P. ostreatus* was firstly reported by Peng *et al.* (1992). Peng *et al.* transformed the homokaryotic strain using the protoplast and electroporation. They used the pAN7-1 vector which is a common vector used in ascomycetes and has a hygromycin selection marker. Yanai *et al.* (1996) reported the transformation using bialaphos selection marker. Kim *et al.* (1999) developed the transformation system using uracil auxotrophic mutant and the corresponding gene. Honda *et al.* (2000) developed the carboxin resistance gene using *in vitro* mutagenesis of iron-sulfur protein subunit of succinate dehydrogenase gene. Currently, Irie *et al.* (2001) reported the genetically modified *P. ostreatus* strain with an expression system for recombinant genes.

## **D. Conservation of genetic resources**

Storage at ultra low temperatures has proved to be the most successful method for the prevention of degenerative changes in filamentous fungi. Therefore, for long term storage, liquid nitrogen storage is generally used for *P. ostreatus*. International Mycological Institute (IMI) reported the successful storage of *P. ostreatus* mycelia in liquid nitrogen for 23 years (Smith, 1993).

## **7. Pests and Diseases**

Although the mushroom itself is a fungus, it can in turn be affected by a range of fungal pathogens, bacterial diseases, viral diseases and insect pests listed as follows:

### **A. Fungal pathogens**

*Pleurotus ostreatus*

*Bolbitius coprophilous* (Peck) Hongo

*Chrysonilia sitophila* (Mont) Arx: Red Bread Mould

*Cladobotryum apiculatum* (Tubaki) W. Gams & Hooz.: Brown Spot, White Soft Rot

*Cladobotryum dendroides* (Bulliard) Merat) W. Gams & Hoozemans: Cobweb, Cobweb

Disease, Cobweb Mould, Mildew, Soft Decay, Soft Mildew

*Cladobotryum variospermum* (Link) Hughes: Cobweb

*Cladosporium* spp.

*Fusarium equiseti* (Corda) Saccardo(1886)

*Fusarium pallidroseum* (Cooke) Saccardo (1886): Pleurotus Wilt  
*Fusarium* spp.  
*Gibberella fujikuroi* (Sawada) Ito (1931): Pleurotus Wilt  
*Gibberella zeae* (Schweinitz) Petch (1936): Pleurotus Wilt  
*Gilmaniella humicola* G.L. Barron  
*Mucor* spp.  
*Penicillium* spp.: Blue-Green Mould, Green Mould  
*Rhizomucor* spp.  
*Trichoderma hamatum* (Bonord) Bain: Green Mould, Grune Schimmel  
*Trichoderma* spp.: Green Mould, Grune Schimmel  
*Verticillium fungicola* (Preuss) Hassebrauk: Dry Bubble, Fungus Spot, Lamole,  
*Verticillium* Brown Spot, *Verticillium* Disease  
*Verticillium* spp.

#### *Pleurotus*

*Aphanocladium album* (Preuss) W.Gams  
*Arthrobotrys pleuroti*  
*Calcarisporium* spp.: Cobweb Disease  
*Cephalotrichum* sp.: Black Mould  
*Chaetomium* spp.  
*Cladobotryum* spp.  
*Coprinus* spp.: Ink Cap, Inky Cap  
*Dactylium* spp.  
*Doratomyces* sp.: Black Mould  
*Mucoraceae* spp.  
*Nigrospora* spp.  
*Peziza* spp.  
*Trichurus* spp.: Black Mould

### **B. Bacterial disease**

#### *Pleurotus ostreatus*

*Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900: Brown Blotch, Mummy Disease  
*Pseudomonas agarici* Young (1970): Brown Blotch, Drippy Gill, Yellow Blotch  
*Pseudomonas fluorescens* Migula 1895 Biovar: Brown Blotch  
*Pseudomonas fluorescens* Migula 1895 Biotype G (=Biovar V): Bacterial Mummy Disease  
*Pseudomonas gingeri* Preece & Wong 1982 (not validly published): Bacterial Blotch,  
 Ginger Blotch  
*Pseudomonas tolaasii* Paine 1919: Bacterial Blotch, Bacterial Brown Blotch, Brown  
 Blotch, Mushroom Blotch

#### *Pleurotus*

*Pseudomonas* spp.: Pseudomonad

### **C. Insect pests**

#### *Pleurotus ostreatus*

*Cyllodes biplagiatus* Le Conte: Beetle

*Hexarthrus davisoni* Waterhouse: Beetle  
*Hypogastrura* (Ceratophysella) *armata* (Nicolet, 1842): Mushroom Springtail, 'Gunpowder Mite'  
*Leiomyza laevigata* Meigen: Fly  
*Leucophenga maculata* (Dufour): Vinegar Fly  
*Lycoriella auripila* (Winnertz): Mushroom Sciarid, Black Fungus Gnat  
*Lycoriella bispinalis* Yang and Zhang: Mushroom Sciarid  
*Lycoriella epleuroti* Yang and Zhang: Mushroom Sciarid  
*Lycoriella jipleuroti* Yang and Zhang: Mushroom Sciarid  
*Lycoriella jingpleuroti* Yang and Zhang: Mushroom Sciarid  
*Lycoriella pleuroti* Yang and Zhang: Mushroom Sciarid  
*Lycoriella yunpleuroti* Yang and Zhang: Mushroom Sciarid  
*Lycoriella* spp.: Black Fungus Gnat  
*Megaselia flavinervis* (Malloch): Mushroom Phorid, Scuttle Fly, Humpbacked Fly  
*Megaselia rubescens* (Wood): Mushroom Phorid, Scuttle Fly, Humpbacked Fly  
*Megaselia* spp.: Mushroom Phorid, Scuttle Fly, Humpbacked Fly  
*Monoclonia* sp.: Fungus Gnats  
*Mycetophila oculus* Walker: Fungus Gnat  
*Mycophila* spp.: Mushroom Yellow Cecid Fly, Gall Midge  
*Mycophila speyeri* (Barnes): Mushroom Yellow Cecid Fly, Gall Midge  
*Oxyporus* (*Pseudoxyporus*) *lateralis* Gravenhorst 1802: Rove Beetle  
*Oxyporus* (*Oxyporus*) *rufipennis* Leconte 1863: Rove Beetle  
*Oxyporus stygicus* Say 1834: Rove Beetle  
*Oxyporus* (*Oxyporus*) *vittatus vittatus* Gravenhorst 1802: Rove Beetle  
*Pheidole nodus* Smith: Ant  
*Phorodonta flavipes* Meigen: Black Fungus Gnat  
*Rhymosia domestica* Meigen: Fungus Gnat  
*Scaphisoma convexum* Say: Beetle  
*Scaphisoma stephani* Leschen and Lobl, 1990: Beetle  
*Sciara fenestralis* Zetterstedt: Fungus Gnat  
*Silvicola cinctus* (Fabricius, 1787): Fly

### *Pleurotus*

*Bleptina* sp.: Moth, Cutworms, Armyworms  
*Cyllodes ater* (Herbst, 1792): Beetle  
*Cyllodes literatus* (Reitter): Beetle  
*Dasytes barbata* (Christoph): Fungus Moth  
*Dasytes rugosella* Stainton: Fungus Moth  
*Heteropezina cathistes* Pritchard: Gall Midge  
*Hydnobioides pubescens* Sen Gupta and Crowson: Beetle  
*Megaselia chaetoneura* (Malloch): Mushroom Phorid, Scuttle Fly, Humpbacked Fly  
*Megaselia frameata* Schmitz: Mushroom Phorid, Scuttle Fly, Humpbacked Fly  
*Megaselia giraudii* (Egger): Mushroom Phorid, Scuttle Fly, Humpbacked Fly  
*Megaselia plurispinulosa* (Zetterstedt, 1960): Mushroom Phorid, Scuttle Fly, Humpbacked Fly  
*Megaselia sylvatica* (Wood, 1910): Mushroom Phorid, Scuttle Fly, Humpbacked Fly  
*Mycomya duplicata* Edwards, 1925: Fungus Gnats  
*Mycetophila ruficollis* Meigen: Fungus Gnat  
*Mycomya marginata* (Meigen, 1818): Fungus Gnats  
*Onthophagus villaneuvai* Delgado-Castillo and Deloya, 1990: Scarab Beetle

*Phanerota dissimilis* (Erichson): Rove Beetle  
*Phanerota fasciata* (Say): Rove Beetle  
*Pleurotobia tristigmata* (Erichson): Rove Beetle  
*Rondaniella* sp.: Fungus Gnat  
*Sciophila lutea* Macquart, 1826: Fungus Gnat  
*Symbiotes* spp.: Beetle  
*Ulodes* spp.: Beetle

#### D. Nematodes

*Pleurotus ostreatus*

Species name not given: Gill Knot Disease  
*Aphelenchoides composticola* Franklin (1957): Mycophagous Nematode  
*Ditylenchus myceliophagus* Goodey (1958): Mycophagous Nematode  
*Paraphalenchus myceliophthorus* Goodey (1958): Mycophagous Nematode  
*Rhabditis axei* (Cobbold) Dougherty (1955): Bacterial Feeding Nematode  
*Rhabditis* spp.: Bacterial Feeding Nematode

#### E. Molluscs

*Pleurotus ostreatus*

*Meghimatium striatum* van Hasselt (1823): Slug

#### F. Mites

*Pleurotus ostreatus*

*Acarus immobilis* Griffith, 1964: Acarid Mite  
*Histiostoma feroniarum* (Dufour, 1839): Bacterial Feeding Mite  
*Proctolaelaps* spp.: Ascid Mite  
*Rhizoglyphus echinopus* (Fumouze et Robin, 1868): Bulb Mite  
*Rhizoglyphus* spp.: Acarid Mite  
*Sancassania* spp. indet: Acarid Mite  
*Tarsonemus* spp.: Tarsonemid Mite  
*Tyrophagus longior* (Gervais, 1844): Seed Mite

#### G. Viruses

*Pleurotus ostreatus*

Partitiviruses and Totiviruses

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## SECTION 12 CAPSICUM ANNUUM COMPLEX

### (Chili peppers, Hot peppers and Sweet peppers)

#### 1. Introduction

*Capsicum annuum* L. is a dicotyledonous flowering plant commonly grown worldwide, with many general names in English, such as hot pepper, chili, chilli or chile pepper, and as well sweet pepper and bell pepper. Sometimes the plant is just called pepper, which however is often reserved for the earlier known Asian *Piper nigrum* (black pepper, white pepper) in the family Piperaceae. The pre-Columbian, indigenous Nahuatl (Aztec) Amerindian name for the plant was transcribed as chilli or chili, and the usual name in Spanish is chile, which results in the plurals of chillies, chilies, and chiles (Bosland 1996). Other broad names for *C. annuum* relate more to particular varieties or strains, culinary uses, and ripeness, such as jalapeño, Cayenne, pimento (pimiento), paprika, red, and green peppers. Furthermore, four other less commonly cultivated *Capsicum* species are also considered chili peppers, and two of these species are similar and closely related to *C. annuum*.

*Capsicum annuum* is usually grown as a herbaceous annual in temperate areas. However, ecologically it is a perennial shrub in tropical areas (which may live a few years to a few decades), and it can be grown as a perennial in climate-controlled greenhouses. This species includes the vast majority of the cultivated pungent and non-pungent (sweet) *Capsicum* peppers in temperate as well as some tropical areas. In the species *C. annuum* throughout the world, there is phenotypic diversity in plant habit and especially in shapes, sizes, colours, pungency, and other qualities of the fruits (Andrews 1995, 1998, 1999; DeWitt and Bosland 1996; Greenleaf 1986). This immense horticultural, agricultural and biological diversity has helped to make *C. annuum* globally important as a fresh and cooked vegetable (e.g. for salads, warm dishes, pickled) and a source of food ingredients for sauces and powders and as a colourant, which is used as well in cosmetics (Andrews 1995, 1999; Bosland 1994; Bosland and Votava 2000). Moreover, the species is used medicinally and medically, and provides the ingredient for a non-lethal deterrent or repellent to some human and animal behaviours (Krishna De 2003; Cordell and Araujo 1993; Palevitch and Craker 1995; Cronin 2002; Cichewicz and Thorpe 1996; Reilly *et al.* 2001). Chili peppers are also cultivated ornamentally especially for their brightly glossy fruits with a wide range of colors.

Chili pepper comprises numerous chemicals including steam-volatile oil, fatty oils, capsaicinoids, carotenoids, vitamins, protein, fibre, and mineral elements (Bosland and Votava 2000; Krishna De 2003). Many chili pepper constituents have importance for nutritional value, flavour, aroma, texture, and colour. The ripe fruits are especially rich in vitamin C (Osuna-García *et al.* 1998; Marin *et al.* 2004). The two chemical groups of greatest interest are the capsaicinoids and the carotenoids. The capsaicinoids are alkaloids that give hot chili peppers their characteristic pungency. The rich supply of carotenoids contributes to chili peppers' nutritional value and colour (Britton and Hornero-Méndez 1997; Hornero-Méndez *et al.* 2002; Pérez-Gálvez *et al.* 2003).

## 2. Taxonomy and Cytology

The genus *Capsicum* L. is in the large family Solanaceae, which includes as food the potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum* or *Solanum lycopersicum*), tree tomato (*Cyphomandra betacea* or *Solanum betaceum*), eggplant (*Solanum melongena*), African eggplants (*Solanum macrocarpon*, *S. aethiopicum*), husk or strawberry tomato (*Physalis pruinosa*) and Cape gooseberry (*P. peruviana*), as well as tobacco (*Nicotiana tabacum*), medicinal plants such as deadly nightshade (*Atropa belladonna*) and *Datura stramonium*, ornamentals such as tree daturas (*Brugmansia*) (which are also hallucinogenic) and *Petunia*, and weeds such as black nightshade (*Solanum nigrum*) (Knapp 2002; Hunziker 2001; George 1985). *Capsicum* is in the subfamily Solanoideae and tribe Capsiceae (Olmstead *et al.* 1999; Knapp 2002; Knapp *et al.* 2004; Hunziker 2001). The genus *Capsicum* consists of about 25 wild and 5 domesticated species (Table 1.30) (IBPGR 1983; Eshbaugh 1993; Bosland and Votava 2000).

The five variously domesticated species are *Capsicum annuum* (Table 1.29), *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubescens* (Heiser and Smith 1953; Smith and Heiser 1957; Heiser 1985). *Capsicum annuum*, *C. frutescens* and *C. chinense* are grouped in a taxonomic complex which has conventionally three, or perhaps two or one species (Pickersgill 1988), with the three clusters of domesticated plants appearing to be more divergent than their wild progenitors (Heiser 1985; Eshbaugh 1993; Prince *et al.* 1995; Idu and Ogbe 1997; Park *et al.* 1999; Bosland and Votava 2000; Walsh and Hoot 2001; Jarret and Dang 2004; Ryzhova and Kochieva 2004; Baral and Bosland 2004). The remaining two domesticated species are in other taxonomic complexes of the genus (Eshbaugh 1993; Tong and Bosland 1999; Walsh and Hoot 2001). Both are little used beyond Latin America, although *C. baccatum* var. *pendulum* (Willd.) Eshbaugh, the variety that has been extensively domesticated, is much used there. For a while, the name *C. frutescens* instead of *C. annuum* was applied to the domesticated chili peppers (Bailey 1923), so in some literature caution is needed to ascertain whether the plants discussed are actually *C. annuum* (which is more likely), or *C. frutescens* itself (*sensu stricto*, i.e. in the narrowly circumscribed sense) or perhaps another of these species (Heiser and Pickersgill 1969; Heiser 1985).

**Table 1.29 Classification of *Capsicum annuum***

Taxonomic placement	Scientific name
Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Solanales
Family	Solanaceae
Genus	<i>Capsicum</i>
Species	<i>annuum</i>
Botanical varieties	var. <i>glabriusculum</i> (synonym var. <i>aviculare</i> ) var. <i>annuum</i>

*Capsicum* species are diploids, with most having 24 chromosomes ( $n = x = 12$ ), but with several wild species having 26 chromosomes ( $n = x = 13$ ) (Table 1.30) (Pickersgill 1991; Tong and Bosland 2003). *Capsicum annuum* has 24 chromosomes; usually 2 pairs (or sometimes 1) are acrocentric, and 10 (or 11) pairs metacentric or sub-metacentric (Lanteri and Pickersgill 1993). Its nuclear DNA content (determined by flow cytometry and Feulgen densitometry) has been reported to have a mean 1C-value of 3.38 picograms per nucleus, which Moscone *et al.* (2003) discuss in relation to other reports with varying methodology that range from 2.76 to 5.07 pg per nucleus. The total length of the chili pepper genome has been estimated to be between 1498 cM and 2268 cM, which is approximately two to three times larger than the tomato genome (Kang *et al.* 2001; Schreiber 2004).

### 3. Centres of Origin and Distribution

The centre of diversity for *Capsicum* is in south-central South America (Eshbaugh 1980; Hunziker 1979; D'Arcy and Eshbaugh 1974; Gonzalez and Bosland 1991; WWF and IUCN 1997), with the majority of species having some range in Brazil and/or Bolivia. Some of the non-domesticated species are gathered for occasional use. The primary centre of origin for domesticated *C. annuum* is in semi-tropical Mexico (Hernández-Verdugo *et al.* 1999, 2001a; Andrews 1995; Long-Solís 1998; Whitmore and Turner 2002). The four other domesticated species are usually believed to have originated in South America (Eshbaugh *et al.* 1983; Walsh and Hoot 2001; Denevan 2001). The centres of origin and domestication of the other two species in the *C. annuum* complex are not as clear (*cf.* Clement 1999). Amazonia (in the northern area) is considered the centre for *C. chinense* ("habanero") (Velez 1991; Toquica *et al.* 2003), and western Amazonia is perhaps the centre for *C. frutescens* ("tabasco"), which is more domesticatedly variable in Central America (Heiser 1985; Hernández-Verdugo *et al.* 1999). Bolivia is considered the centre of domestication for *C. baccatum* (aji) (in the subtropical east) and *C. pubescens* (rocoto) (in the mid-elevation Andes, where known only in cultivation) (Eshbaugh *et al.* 1983; Eshbaugh 1993).

By molecular analysis, Loaiza-Figueroa *et al.* (1989) confirmed that the centre of domestication of *C. annuum* var. *annuum*, the cultivated variety, is the upland region (Sierra Madre Oriental) of central-eastern Mexico (in the states of Nuevo León, Tamaulipas, San Luis Potosí, Veracruz and Hidalgo). Its ancestor probably is the wild chiltepín (bird pepper), *C. annuum* var. *glabriusculum* (Dunal) Heiser & Pickersgill, which has a range of unclear natural extent from southern USA through Mesoamerica to Colombia and the Caribbean, and is sometimes wild-harvested still and semi-domesticated as well (Eshbaugh 1993; Hernández-Verdugo *et al.* 2001a; Votava *et al.* 2002; Vásquez-Dávila 1996; Guzmán *et al.* 2005).

The earliest archaeological evidence of *Capsicum* being used dates to 10,500 BP (*C. baccatum*) and 10,000-9,500 BP (*C. chinense*) in the western Central Andes of Peru (Brack 2003; Smith 1980). Substantial evidence in Peruvian dry coastal river valleys, with specimens increasing from rare to moderately abundant through eight millennia, indicates expanding cultivation and domestication of *Capsicum* (Pearsall 2003). *Capsicum frutescens* is recognised in the northwestern area by 4400-3200 BP (Brack 2003). Intriguingly, *Capsicum* seems to be absent from the more recent Chiribaya culture of 1100-600 BP in far southern Peru (Flamini *et al.* 2003), even though earlier (2200-1400 BP) in southwestern Peru *C. frutescens* was being used. The importance of *Capsicum* is suggested by an obelisk from Chavín de Huántar ( $\approx$  2800 BP) in the north-central Peruvian Andes, featuring a foundational Earth-crocodilian associated with (apparently) gourd, chili pepper, manioc (cassava) and peanut (Brotherston 1979; Miller and Burger 1995). Similarly, there is archaeological evidence from about 9000 BP for the use and subsequent domestication of *Capsicum annuum* in central-eastern and south-central Mexico in the states of Tamaulipas (near Ocampo), Puebla (Tehuacán Valley) and Oaxaca (Guilá Naquitz) (Pickersgill 1984; Bosland 1996; *cf.* Smith 2001, 2005).

*Capsicum* was brought to Europe by Columbus in 1493 as the peppery spice that signified the success of his quest, and the early European voyagers to the Caribbean, Mesoamerica and South America encountered a plethora of variety and landraces of this common food as well as medicinal plant (Sauer 1966; Long-Solís 1998). The ready appeal of *Capsicum* was such that within half a century it had been distributed as far as Asia, and it has been integrated and continues to be diversified in cultures worldwide as it had been originally in the Americas (Ferrão 1992; Andrews 1992, 1995, 1998, 1999; DeWitt and Bosland 1996; Eshbaugh 1983; Yamamoto and Nawata 2004, 2005).

The species of *Capsicum* were listed with their seemingly natural distributions by the International Board for Plant Genetic Resources (IBPGR 1983) and updated by Eshbaugh (1993), Hernández-Verdugo *et al.* (1999) and Bosland and Votava (2000); they are listed in Table 1.30 as currently understood. There is modest uncertainty on the generic limits of *Capsicum* and more uncertainty on its tribal relatives (which at minimum include *Lycianthes*) (Eshbaugh 1993; Hunziker 2001; Knapp 2002), and a lack of consensus on the number and in a few cases the botanical names of the known *Capsicum* species, and on the truly natural distributions of several species (rather than confounding naturalised with native populations).

**Table 1.30 The species of *Capsicum* and their known or apparently natural distributions; those with haploid chromosome number  $n=13$  rather than  $n=12$  are noted (Tong and Bosland 2003). The five domesticated species are grouped into the *C. annuum* complex (3 spp.) (CA), the *C. baccatum* complex (CB), and the *C. pubescens* complex (CP) (Tong and Bosland, 1999; Walsh and Hoot, 2001; Jarret and Dang, 2004; Ryzhova and Kochieva, 2004)**

Species	Known or probable natural distribution
<i>C. annuum</i> L. (CA)	southern USA to Colombia
<i>C. baccatum</i> L. (CB)	Peru, Bolivia, Paraguay, Argentina and Brazil
<i>C. bulfurum</i> Hunz.	southern Brazil
<i>C. campylopodium</i> Sendtner; $n = 13$	southern Brazil
<i>C. cardenasii</i> Heiser & P.G. Smith (CP)	northeastern Bolivia
<i>C. chacoense</i> Hunz. (CB or CA)	Argentina, Paraguay and Bolivia
<i>C. chinense</i> Jacq. (CA)	(northern) Amazonian South America
<i>C. coccineum</i> (Rusby) Hunz.	Bolivia and Peru
<i>C. cornutum</i> (Hiern) Hunz.	southern Brazil
<i>C. dimorphum</i> (Miers) Kuntze	Colombia
<i>C. dusenii</i> Bitter	southeastern Brazil
<i>C. eximium</i> Hunz. (CP)	Bolivia and northern Argentina
<i>C. flexuosum</i> Sendtner	Argentina, Brazil and Paraguay
<i>C. frutescens</i> L. (CA)	western Amazon (Colombia to Peru)
<i>C. galapagoense</i> Hunz. (CA)	Galápagos Islands (Ecuador)
<i>C. geminifolium</i> (Dammer) Hunz.	Colombia and Ecuador
<i>C. hookerianum</i> (Miers) Kuntze	Ecuador and northwestern Peru
<i>C. lanceolatum</i> (Greenman) Morton & Standley; $n = 13$	Honduras, Guatemala and Mexico
<i>C. leptopodium</i> (Dunal) Kuntze	Brazil
<i>C. minutiflorum</i> (Rusby) Hunz.	Argentina, Paraguay and Bolivia
<i>C. mirabile</i> Mart. ex Sendtner; $n = 13$	southern Brazil
<i>C. parvifolium</i> Sendtner	northeastern Brazil, Venezuela and Colombia
<i>C. praetermissum</i> Heiser & P.G. Smith (CB)	southern Brazil
[synonym <i>C. baccatum</i> var. <i>praetermissum</i> (Heiser & P.G. Smith) Hunz.]	
<i>C. pubescens</i> Ruiz & Pavón (CP)	Bolivia to Colombia [only in cultivation]
<i>C. rhomboideum</i> (Dunal) Kuntze	Mexico to Peru
[synonym <i>C. ciliatum</i> (Kunth) Kuntze]; $n = 13$	
<i>C. schottianum</i> Sendtner; $n = 13$	southern Brazil, Paraguay and Argentina
<i>C. scolnikianum</i> Hunz.	northwestern Peru
<i>C. tovarii</i> Eshbaugh, P.G. Smith & Nickrent (CB)	south-central Peru
<i>C. villosum</i> Sendtner	southern Brazil

#### 4. Morphological Characters and Molecular Markers

##### A. Morphological characters for identification

The five domesticated species are differentiated by using morphological characters that rely primarily on colour and morphology of flowers and seeds (Andrews 1995; DeWitt and Bosland 1996),

as shown in Table 1.31. However, identifying some plants in the diverse *C. annuum* complex can be problematic (Pickersgill *et al.* 1979; Bosland and Votava 2000; Jarret and Dang 2004; Baral and Bosland 2004). Capsaicinoid profiles are not reliable as unique indicators for identification, though the profile may be useful as a supplementary character. In one study (7-58 accessions per species), the accuracy of identification based solely on capsaicinoid profiles, in the *C. annuum* complex, was 82% of the *C. chinense* accessions, 57% for *C. annuum* and just 20% for *C. frutescens* (but its sample was only 10 accessions), and similarly was 59% for *C. baccatum* and 86% for the distinctive *C. pubescens* (Zewdie and Bosland 2001).

**Table 1.31 Morphological characters that generally differentiate the domesticated species of *Capsicum***

Species	Flowers per node	Calyx	Corolla colour	Corolla lobe basal spots	Anther colour	Seed colour
<i>C. annuum</i> var. <i>annuum</i>	1 (-5)	no ring; often teeth	white to dingy white (rarely purple)	none	blue-purple	straw (tan)
<i>C. frutescens</i>	usually 2-4 (1-6)	no ring; usually no teeth	greenish white or greenish	none	blue-purple	straw (tan)
<i>C. chinense</i>	(1-) 2 (-5)	annular ring; no teeth	greenish white or white	none	blue	straw (tan)
<i>C. baccatum</i> var. <i>pendulum</i>	1 (-2)	no ring; teeth	white (cream) or greenish-white	yellow-green	white to yellowish	straw (tan)
<i>C. pubescens</i>	1	no ring; teeth	purple or purple-white	none	purple (purple-white)	black (brown/black)

Source : after Lippert *et al.*, 1966; Heiser, 1985; Greenleaf, 1986; Eshbaugh, 1993; Jarret and Dang, 2004

Cultivated *Capsicum annuum* var. *annuum* is very diverse regionally and worldwide (*e.g.* DeWitt and Bosland 1996; Andrews 1995, 1998), having a wealth of innumerable strains, landraces and varieties that defy both facile description (IPGRI *et al.* 1995; Zewdie *et al.* 2004) and clustering into an inclusive and practicable classification (Bosland and Votava 2000). Sometimes typical characteristics (fruit shape, size, and pungency) have been featured and organised, recognising the Cerasiforme Group (cherry peppers), Conoides Group (conical peppers), Longum Group (*e.g.* Cayenne peppers) and Grossum Group (blocky sweet or bell peppers) (*cf.* Mabberley 1998; Bailey 1923), but as more plants are considered the array of variations and combinations of notable traits increases (*e.g.* fruit sizes and shapes intermediate, whether fruits are erect or pendent), and the groups become less distinct and meaningful.

## B. Molecular markers

Various molecular markers have been used for identification of chili peppers, and to evaluate their germplasm diversity. A review is provided by Lefebvre (2005). Rodriguez *et al.* (1999) found diagnostic RAPD (randomly amplified polymorphic DNA) markers for four of the domesticated species (and *C. chacoense*), but not for *C. frutescens*. Use of isozymes has focused predominantly on measuring genetic variability, and clarifying phylogenetic relationships within the genus (Eshbaugh 1993). Conicella *et al.* (1990) analysed esterase isozymes in 15 accessions of *C. annuum* from the Americas; these data plus cytological data also provided evidence that Mexico is the centre of domestication for *C. annuum*.

Prince *et al.* (1995) utilised RAPDs in studying molecular polymorphism in *C. annuum*. Lefebvre *et al.* (2002) developed an integrated intraspecific *C. annuum* molecular linkage map using phenotypic and isozyme markers, known functional genes, RAPDs, RFLPs (restriction fragment length

polymorphisms) and AFLPs (amplified fragment length polymorphisms) from F<sub>1</sub> and F<sub>2</sub> hybrids derived from double-haploid *C. annuum* populations. Using RAPDs and AFLPs on a broad array of *C. annuum* types (from 34 accessions of nine countries), Paran *et al.* (1998) separated large-fruited sweet peppers from small-fruited pungent peppers, and found more genetic variation among the pungent cultivars. Tam *et al.* (2005) found the SSAP (sequence-specific amplification polymorphism) marker system generally more informative than using AFLPs or SSRs (simple sequence repeats or microsatellites). They had similar overall results between large-fruited sweet (bell) types and conical types, but within their pungent as well as sweet conical types the grouping of some cultivars shifted depending on which of the three marker systems was employed.

Tanksley (1984b) developed the first linkage map of *Capsicum* by studying segregating isozymes in an interspecific cross between *C. annuum* and *C. chinense*. Genomic similarities and differences between *Capsicum* and *Lycopersicon* were studied by Tanksley *et al.* (1988) to construct the first RFLP linkage map of chili pepper (with 85 loci). A more complete map was developed by Prince *et al.* (1993), and Livingstone *et al.* (1999) provided a still more comprehensive comparative map with over 1000 loci using mainly AFLP and RFLP markers. Repetitive DNA sequences make up a maximum of 10% of the chili pepper genome, which overall is at least four times more copies than in tomato (Schreiber 2004). Kang *et al.* (2001) constructed a more complete interspecific (*C. annuum* × *C. chinense*) F<sub>2</sub> molecular linkage map using mainly pepper-derived RFLP probes and AFLP markers, and Lee *et al.* (2004a) augmented the map using SSRs. Paran *et al.* (2004) constructed an integrated genetic linkage map of these *Capsicum* spp., consisting of 2262 diverse markers (including several known gene sequences) and covering 1832 cM and 13 linkage groups (with only 15 gaps > 10 cM remaining). An RFLP-based map (92 markers) of an interspecific (*C. annuum* × *C. frutescens*) BC<sub>2</sub> population has also been constructed (Rao *et al.* 2003).

## 5. Reproductive Biology and Ecology

### A. Reproductive organs (morphology, development), fertilisation, dispersal and germination

#### *Flower*

*Capsicum annuum* starts flowering at the axil of the first branching node, with subsequent flowers forming at each additional node (Bosland and Votava 2000). Usually *C. annuum* has a solitary flower at the axil (Bosland and Gonzalez 1994), although some accessions have a few clustered flowers between which there are short internodes (Smith and Heiser 1951) (Table 1.31). Flower differentiation is not affected by daylength (Cochran 1942). The most important factor determining differentiation is air temperature, especially at night (Bosland and Votava 2000; Aloni *et al.* 1999; Rylski 1972).

The *Capsicum* flower is bisexual, hypogynous and usually pentamerous (Bosland and Votava 2000). The flowers are complete, with calyx, corolla, and male and female sex organs. The diameter of a *C. annuum* flower is 9-15 mm. The *Capsicum* calyx is broadly campanulate, ribbed, about 2 mm long, and truncate or undulate to weakly or prominently dentate with 5-7 teeth. The short-tubed corolla is rotate in most *Capsicum* species, with usually 5 but sometimes 6-7 (-8) petals in some species. The number of corolla lobes and stamens is equal. Typically the flowers have 5 stamens; the filaments are white or violet depending on the species (or variety), with the usually connivent to free anthers varying from bluish-purplish to yellow and white depending on the species (*e.g.* Table 1.31) (Dharamadhaj and Prakash 1978). The pistil comprises an ovary of 2-3 (-4) carpels that is 2-5 mm long and 1.5-5 mm in diameter, a style 3.5-6.5 mm long, and a capitate papillate stigma slightly wider

than the style. The style extends well beyond to just beyond the anthers or may be even with them, or it may be slightly exceeded by the anthers.

The daily start of anthesis apparently is controlled by daylength (Aleemullah *et al.* 2000). The corolla typically opens within the first 3 hours after sunrise, and the petals remain open for less than a day; there also can be a smaller peak of anthesis in the afternoon. Hirose (1957) found dehiscence of the anthers to occur late in the morning, between 10 am and noon. The anthers open lengthwise from typically 1 hr after the flower opens to even 10 hrs afterward, but they frequently fail to dehisce entirely, or may dehisce the next morning if the flower opens in late afternoon (Aleemullah *et al.* 2000; *cf.* Horner and Wagner 1992). Depending on the environmental conditions and variety, the period of receptivity of the stigma is 5-8 days, from several days before anthesis to fewer days afterwards, with maximum fertility on the day of anthesis (Cochran and Dempsey 1966; Barai and Roy 1986; Aleemullah *et al.* 2000).

#### *Pollen and fertilisation*

The pollen grains of chili pepper are medium to light yellow, subspheroidal, pitted, and tricolporate with longitudinal grooves (Bosland and Votava 2000). The plant has about 1-1.5 mg of pollen per flower (Quagliotti 1979), with 11,000-18,000 pollen grains in a single anther (Hirose 1957). Air temperature has a large effect on pollen formation and viability. Temperatures above 30°C occurring 15 days prior to anthesis cause pollen sterility (Cochran 1938), and night temperatures of 10 ± 2 °C reduce the number and germinability of pollen grains (Shaked *et al.* 2004). The optimal temperature for pollen germination is 20-25°C. Pollen tube growth from the stigma to the egg has been reported to take 6 to 42 hrs. In detailed anatomical studies, Cochran (1938) found that fertilisation occurred 42 hrs after pollination in plants grown at 27°/21°C, whereas Kato (1989) found that 36 hrs were needed for the fertilisation process.

Male sterility is found in *Capsicum*, controlled by cytoplasmic and nuclear genes (Shifriss 1997; Wang *et al.* 2004; Kalloo *et al.* 2002). In plants of both types, the anthers may be small and shrunken and blue-violet, with little or no viable pollen (Wien 1997), or there may be no anthers (Derera *et al.* 2005).

#### *Fruit*

There is extensive diversity in fruit shape, size, wall thickness and fleshiness, colour and pungency (Andrews 1995; IPGRI *et al.* 1995), determined by genetic and environmental factors. Among the innumerable varieties of *C. annuum*, the diversification of shapes of the pod (fruit) is striking — *e.g.* blocky (or lantern- or bell-shaped), globose, oblong (sausage-shaped), ovoid, conical, cylindrical, banana-like (curved); and smooth, grooved, lumpy or wrinkled. The length of the pod varies from less than 1 to 32.5 cm. The pedicel length also varies in different pod types (over several cm), and the fruit may be erect to pendent (deflexed). Fruit colours range from green, yellow, orange and red to purple, brown, black, and white as well. Some of the genetics of fruit colour and shape are becoming well understood (Ben Chaim *et al.* 2003; Thorup *et al.* 2000; Huh *et al.* 2001).

Morphologically the *Capsicum* fruit is a berry, sometimes with a few stone cells (sclerified inclusions in the fleshy portion) (Knapp 2002). The pericarp consists of epidermal cells in regular order with a thick-grooved cuticle. Several rows of collenchymatously thickened beaded cells constitute the hypodermis. The mesocarp is formed by thick-walled beaded cells; the inner mesophyll cells are thin-walled ground parenchyma and fibrovascular bundles. Giant cells (perhaps unique to *Capsicum*) occur on the inner wall of the endocarp (Fridvalsky and Nagy 1966). The vascular bundles consist of xylem tissue with spiral vessels and phloem tissue. The pod has two, three or four locules,

with each corresponding wall of the axile placenta having vesicles for production of capsaicinoids (Suzuki *et al.* 1980).

Usually there are many more flowers than fruits (Marcelis *et al.* 2004). The most obvious sign of assimilate competition or dominance among the organs is abscission of flowers and small fruits during the most active fruit-growth period, resulting in a cycling of flowering and fruit set (Hall 1977; Clapham and Marsh 1987; Bhatt and Srinivasa Rao 1997; Marcelis *et al.* 2004). The most actively growing organ of a chili pepper plant after flowering is the fruit (Hall 1977; Beese *et al.* 1982; Marcelis and Baan Hofman-Eijer 1995). Fruit growth is dependent on ovule growth (whether fertilised). The fruit is ordinarily seeded, but parthenocarpic forms exist (Heuvelink and Körner 2001). The seed set affects development and subsequent growth of the fruit; on average there is a direct linear relationship between the number of seeds per fruit and final fruit size, until saturation at perhaps over 200 seeds per fruit (Marcelis and Baan Hofman-Eijer 1997). The number of seeds per fruit ranged from 1 to 34 in wild northwestern Mexico populations of *C. annuum* (Hernández-Verdugo *et al.* 2001b). A low of 50-100 seeds per cultivated fruit (20-30% of maximum) is sufficient for maximal fruit set (Marcelis and Baan Hofman-Eijer 1997); blocky sweet pepper (bell pepper) may average 150-300 seeds per fruit (Aloni *et al.* 1999).

The time from anthesis to a fully grown fruit varies considerably among different pod types (Bosland and Votava 2000). Typically cultivated fruit reaches the mature green stage in 35-50 days after the flower is pollinated. This stage is horticulturally ripe for some uses, but still physiologically immature. Fruit maturity depends on the cultivar, and the environmental conditions before and during maturation (Perry *et al.* 1993; Montes Hernández *et al.* 2004). The fruits are characterised as non-climacteric in ripening (Lownds *et al.* 1993), apparently lacking the typical increase in CO<sub>2</sub> and ethylene production as they ripen (Saltveit 1977).

The fruits of most *Capsicum* are pungent, because the placenta accumulates capsaicinoids (*e.g.* capsaicin) (Zewdie and Bosland 2001; Thompson *et al.* 2005), except in domesticated non-pungent (sweet) varieties which are mostly developed in *C. annuum* (Bosland and Votava 2000). The pungency trait is controlled at a single locus on chromosome 2; when the pungency gene *Pun1* (also called *C*) is homozygous recessive (*i.e.* present as *pun1/pun1* or *cc*), the capacity to make capsaicinoids is lost (Stewart *et al.* 2005; Blum *et al.* 2002). In the pungent chili peppers, other genes variously affect the synthesis of capsaicinoids (Blum *et al.* 2003), and production is also affected by physiological interactions and the environment (Zewdie and Bosland 2000a; Estrada *et al.* 2002; Sung *et al.* 2005). The individual fruit's pungency (content of capsaicinoids) is affected by its node position on the plant, whereas its capsaicinoid profile remains fairly constant (Zewdie and Bosland 2000b; Estrada *et al.* 2002; Kirschbaum-Titze *et al.* 2002). Capsaicinoids increase with fruit growth to a maximum (*e.g.* 40-50 days after fruit set), then decline (Contreras-Padilla and Yahia 1998). Capsaicinoids can be transported within the plant, with different capsaicinoid profiles found in stems and leaves (Estrada *et al.* 2002).

#### *Fruit dispersal*

The red fruits of wild *C. annuum* var. *glabriusculum* attract birds, which eat them and disperse viable seeds, but their pungency discourages consumption by wild mammals (Vásquez-Dávila 1996; Tewksbury *et al.* 1999; Tewksbury and Nabhan 2001). Rats experimentally fed hot chili peppers for 2-11 months became desensitised to aversion, but indifferent rather than developing a preference for this spicy food (Rozin *et al.* 1979). Nonetheless, the widespread and common little yellow-shouldered bat (*Sturmira lilium*), which sometimes favors solanaceous fruits (Passos *et al.* 2003; Galindo-González *et al.* 2000), has been reported to consume pungent *Capsicum* in northwestern Argentina and

disperse the seeds — which is favored by local people who recognise this increases the number of wild plants, as they gather the fruits for home seasoning and village marketing (Iudica 1999).

#### *Seed and germination*

The seed develops from a campylotropous ovule (Dharamadhaj and Prakash 1978). Within a pod, the many seeds are attached to the placenta walls in close rows, mainly near the calyx end. The seeds are disk-like with a deep chalazal depression. The embryo is surrounded by a well-defined endosperm which makes up the bulk of food reserves for the embryo and young seedling. The endosperm lies directly in front of the radicle and consists of seven to nine thick cells (Watkins *et al.* 1985). *Capsicum annuum* seeds have mainly protein and lipids as storage reserves (Chen and Lott 1992). The seed is covered by a parchment-like seed coat, which does not cause a mechanical restriction to germination (Watkins and Cantliffe 1983b). Seed colour inheritance involves at minimum about three genes (Zewdie and Bosland 2003). Seed size is somewhat dependent on the variety and growing conditions. Seed mass maturity may occur about 50 days after anthesis, with 10-12 more days required for maximum potential longevity but 17-21 days for maximal seedling dry weight (based on variation in time from sowing to emergence) (Demir and Ellis 1992). An average *C. annuum* seed is about 5.3 mm long, 4.3 mm wide and 1 mm thick, with a surface area of 33 mm<sup>2</sup> (Chen and Lott 1992).

Freshly harvested seeds of certain wild *Capsicum* species can exhibit dormancy (Bosland and Votava 2000; Wien 1997; IBPGR 1983). An after-ripening period at room temperature may be required to remove dormancy (Randle and Homna 1981). As *C. annuum* seeds age and lose viability (Ozocoban and Demir 2002) they may become brown. Seed dormancy may be broken by treatment with 0.2 M KNO<sub>3</sub> under white light (750-1250 lux) and alternating temperatures (30°/20°C or 30°/15°C) (*cf.* Hernández-Verdugo *et al.* 2001b). Seeds of cultivated *C. annuum* can be cryopreserved at -196°C and moisture content of 4.7-11.5%, and subjected to rapid or slow freezing and thawing (Quagliotti and Comino 2003).

*Capsicum* species seeds germinate well in a constant temperature range between 15°C and 30°C (Randle and Homna 1980; *cf.* Dell'Aquila 2004), and do not germinate when exposed to temperatures below 8°C or above 40°C (Choi 1985). No special light requirements are necessary for germination of domesticated chili pepper seeds, whereas seeds of wild *C. annuum* var. *glabriusculum* do not germinate in constant darkness (Hernández-Verdugo *et al.* 2001b).

### **B. Sexual reproduction**

#### *Pollination*

*Capsicum* species are usually self-compatible (Onus and Pickersgill 2004), and *C. annuum* is a partially self-pollinating crop (Allard 1960; Rylski 1986); wind or similar mechanical disturbance may enhance self-pollination (Raw 2000; Kristjansson and Rasmussen 1991). Outcrossing is associated with insect pollinators, less with wind (Odland and Porter 1941; Tanksley 1984a; Raw 2000). The proportion of plants cross-pollinated depends on several factors and can range from 2 to 90% (Pickersgill 1997); in many localities, cross-pollination is predominant. The effect of outcrossing on fruit set of *C. annuum* is significant. Nagarathnam and Rajamani (1963) obtained only 6-11% fruit set when flowers were isolated to self-pollinate. Erwin (1937) found that 46% of self-pollinated flowers set fruit, compared to 71% for flowers left to open-pollinate by bee activity. In field research *Capsicum* should be considered facultative cross-pollinating species (Odland and Porter 1941; Tanksley 1984a). Breeders and seed producers thus need to undertake precautionary measures to prevent uncontrolled cross-pollination (Bosland 1993). To produce large amounts of genetically pure seeds, seed certification programmes employ isolation as the control mechanism. Isolation

requirements may range from 400 m for the Certified class to 1.6 km for the Foundation class (NMCIA 1992) but depend on local conditions, for example being 300 m in Hungary but perhaps requiring 2-3 km or more in Australia (Derera *et al.* 2005).

The odourless flowers are visited by insects both for sugary nectar, which is mostly hexoses and low in daily amount (greatest on the day of anthesis), and also for their pollen (Rabinowitch *et al.* 1993; Vogel 1998; Roldán-Serrano and Guerra-Sanz 2004; Raw 2000). Solitary bees, honeybees, bumblebees, aphids and thrips are likely to transfer the pollen grains, especially those that obtain pollen by buzz pollination, shaking the anthers (Andrews 1995; Raw 2000; de Ruijter *et al.* 1991; Kubišová and Háslbachová 1991; Shipp *et al.* 1994; Dag and Kamer 2001; Kristjansson and Rasmussen 1991).

#### *Crossability and hybridisation*

*Capsicum* species do not hybridise with species in other genera of the Solanaceae (Berke 2000). Pepper breeding continues to be highly rewarding for the improvement of *Capsicum* (Poulos 1994; Berke 2000; Geleta and Labuschagne 2004). Interspecific crossing between many *Capsicum* species has been tried experimentally (often repeatedly) for agronomic and taxonomic purposes (*cf.* Walsh and Hoot 2001; Pickersgill 1991, 1997; Onus and Pickersgill 2004). Fertile hybridisations can occur between taxa within the *Capsicum annuum* complex to varying degrees (Jarret and Dang 2004; Nwankiti 1976; Kumar *et al.* 1987; Panda *et al.* 2004; Baral and Bosland 2004), and also these species with *C. baccatum* but not with *C. pubescens*; Table 1.32 below gives a synopsis (*cf.* Yoon *et al.* 2004). Similar interspecific spontaneous or natural hybrids of these species are difficult to ascertain, but infrequently surmised (Jarret and Dang 2004; Rodriguez *et al.* 1999). Their recognition is confounded by taxonomic uncertainty, the extensive variability from selection within the domesticated species for millennia to decades, and the plasticity of individual plants. Crossings between wild and semi-domesticated *C. annuum* var. *glabriusculum*, and between feral or weedy and domesticated *C. annuum* var. *annuum*, and these two complexes hybridising with each other, are probably a regular occurrence and vary in fertility (Jarret and Dang 2004; Guzmán *et al.* 2005; Hernández-Verdugo *et al.* 2001a; Prince *et al.* 1992; Pickersgill 1971). Crossing also is probable in many regions in the tropics between cultivated and feral *C. frutescens* (*e.g.* Yamamoto and Nawata 2004, 2005; Symon 1981; Wiggins and Porter 1971).

**Table 1.32 Crossability (including hybrid viability) of *Capsicum annuum* with other *Capsicum* in the three complexes of domesticated species; see Table 1.30**

<i>Capsicum annuum</i> (C <sub>A</sub> ) reciprocal crosses with other <i>Capsicum</i> species	
Interspecific parent and species complex	<i>Capsicum</i> sp. as female / male
<i>C. frutescens</i> (C <sub>A</sub> )	(+) / +
<i>C. chinense</i> (C <sub>A</sub> )	(+) / (+)
<i>C. galapagoense</i> (C <sub>A</sub> )	† / +
<i>C. chacoense</i> (C <sub>B</sub> or C <sub>A</sub> )	0 / +
<i>C. baccatum</i> (C <sub>B</sub> )	+ / +
<i>C. praetermissum</i> (C <sub>B</sub> )	† / †
<i>C. tovarii</i> (C <sub>B</sub> )	0 / 0
<i>C. pubescens</i> (C <sub>P</sub> )	0 / 0
<i>C. cardenasii</i> (C <sub>P</sub> )	0 / †
<i>C. eximium</i> (C <sub>P</sub> )	0 / †

F<sub>1</sub> hybrids produce: viable seeds +, or some viable seeds (+); non-viable seeds †; or no fruits and/or seeds 0.

Source : after Pickersgill, 1971; IBPGR, 1983; Zijlstra *et al.*, 1991; Tong and Bosland, 1999

### C. Asexual reproduction

The chili pepper plant can be propagated asexually by means of cuttings and grafting. Young cut shoots form whole independent plants with roots *in vitro* as well as in the field (Choi *et al.* 1999; Shirai and Hagimori 2004). Scions from chili pepper plants graft successfully on stocks of chili pepper (Chung and Choi 2002) as well as tomato (Deloire and Héban 1982). The grafted plants can set flowers and fruits. *Capsicum* grafting can induce genetic changes, which may provide variations of breeding value (Taller *et al.* 1998, 1999).

## 6. Crop Production and Use

Chili peppers are grown worldwide, either outside in fields or in greenhouses. The ability to produce a quality crop in such a wide range of climates and conditions has helped to make chili pepper a globally common crop. Because of the extensive cultivation, adaptation and variability of *C. annuum*, it is difficult to generalise to what is typical, and there is no single method for production (Bosland and Votava 2000).

### A. Environmental conditions

Chili pepper is a warm-season crop (Rodríguez-Rey *et al.* 2000), and highly susceptible to frost. Watkins and Cantliffe (1983a) showed that at 25°C radicle emergence required 3.5 days, whereas at 15°C, 9 days were required. Seedling emergence from a soil depth of 1.2 cm took 8-9 days at temperatures from 25-35°C (Lorenz and Maynard 1980), but was prevented below 15°C (Wien 1997). The leaf unfolding rate of seedlings (based on maximum leaf count), which is also a measure of node and internode formation, was optimal at an average daily temperature of ≈ 26°C (Si and Heins 1996).

The plant is usually indeterminate and has continuous sympodial branching, with the individual branch systems apparently functioning as relatively autonomous integrated physiological units (Thomas and Watson 1988; *cf.* de Swart *et al.* 2004). For a high yield of good quality fruit, Bakker and van Uffelen (1988) found that mean air temperatures of 21-23°C were optimal during vegetative growth, followed by 21°C during fruit growth. The minimum temperature for growth and development is 18°C, below which growth is trivial, with plants in the 5-15°C range growing poorly (Sanders *et al.* 1980). The most growth in the vegetative stage occurs at 25-27°C day temperature and 18-20°C night temperature (Dorland and Went 1947; Bakker and van Uffelen 1988). Day temperature lower than

night temperature is detrimental to vegetative growth (as is a low night temperature of 12°C). Nonetheless, to grow compact greenhouse seedlings, higher night temperature is preferable (Si and Heins 1996; Sysoyeva and Kharkina 2000).

Maximum flower set occurs when day and night temperatures are between 21°C and 16°C. Flowers drop when the night temperature is above 24°C. Yields are high when the daily air temperature during fruit set ranges between 18-32°C. Fruits do not set when the mean daily temperature is above 32°C, or is below 16°C — or when cooler, the fruits are malformed (Olareweju 1988; Aloni *et al.* 1999). Productivity is constrained by the adverse effects of high temperature on fruit set, and the detrimental influence of low temperature on fruit shape (Rylski and Spigelman 1982; Rylski 1973).

### **B. Agricultural practices**

The ideal soil for producing chili pepper is deep, well-drained, medium-textured sandy loam or loam that holds moisture and has some organic matter. Plants can be started by direct seeding, or by transplanting after initial growth in trays (Bosland and Votava 2000); the plants are started in greenhouses or hotbeds in many production areas, or in outdoor seedbeds in mild-climate areas. Chili pepper plants are transplanted when they are 6-8 weeks old. Prior to field planting, the plants should be hardened but not excessively.

Whether the field population is established by transplanting or direct seeded, the optimum crop is dependent upon row spacing and between-row spacing of the plants, and the type grown (Bosland and Votava 2000). Chili peppers require adequate amounts of most major and minor nutrients; the most-utilised are normally N and P. Plastic mulch maintains moisture in the soil; increases soil temperature and early yields; reduces weed populations, fertiliser leaching and soil compaction; and protects fruits from soil deposits and soil microorganisms. Competition between weeds and chili peppers for nutrients, light and water is a serious problem in production (Lee and Schroeder 1995). A successful weed control programme is essential for producing a healthy crop. Abiotic stresses include extreme temperatures, moistures, light, nutrients, pH, pollutants and pesticides.

Row covers or tunnel planting systems have been used for production in the field because of their effectiveness to alter microclimates. Chili pepper is sensitive to excessive water (Suh *et al.* 1987). Irrigation is not necessary in areas with regular and ample rain, although it generally is essential in arid and semi-arid regions. Chili pepper is a shallow-rooted crop (González-Cervantes *et al.* 2004). The amount and frequency of irrigation depend on soil type, bed type, plant size, humidity, wind, sunlight and prevailing temperatures. A limited supply of water during the rapid vegetative-growth period reduces the final yield (Beese *et al.* 1982; Srinivasa-Rao and Bhatt 1988; Sato *et al.* 2003). Fruits grown under water deficit may have a higher concentration of capsaicin (Sung *et al.* 2005).

Chili pepper plants can be made to behave perennially under greenhouse conditions, with environmental control carried out by air temperature regulation, supplemental light, and CO<sub>2</sub> enhancement as well. Regular removal of flowers leads to faster vegetative growth (Hall 1977; Clapham and Marsh 1987). In The Netherlands non-pungent chili peppers are greenhouse-grown on 1200 ha, and about a third of the workers develop an allergy to the pollen, which can be alleviated by introducing honeybees to remove pollen (Blacquière *et al.* 2004).

### **C. Biotic stresses**

Production can be diminished by various biotic stresses. Chili pepper is susceptible to diseases and pests that can be primary constraints on cultivation (Bosland and Votava 2000; DeWitt and

Bosland 1993), and their control is one of the most important factors in producing a profitable crop. The diseases and pests usually reduce both quality and quantity of fruits.

Diseases from bacteria infecting the chili pepper plants include bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*), bacterial canker (*Corynebacterium michiganense* or *Clavibacter michiganensis* subsp. *michiganensis*), bacterial soft rot (*Erwinia carotovora* pv. *carotovora*) and bacterial wilt (*Pseudomonas solanacearum* or *Ralstonia solanacearum*). The plants are susceptible to fungi which cause diseases such as anthracnose (*Colletotrichum* spp.), early blight (*Alternaria solani*), Cercospora leaf spot (*Cercospora capsici*), damping-off/seedling disease (*Pythium*, *Rhizoctonia*, *Fusarium*, etc.), Fusarium stem rot (*Fusarium solani*), grey mold (*Botrytis cinerea*), Phytophthora blight and root rot (*Phytophthora capsici*), powdery mildew (*Leveillula taurica* or *Oidiopsis taurica*), Rhizoctonia root rot (*Rhizoctonia solani*), Stemphylium leaf spot (*Stemphylium botryosum* f. sp. *capsicum*), gray leaf spot (*Stemphylium solani* and *S. lycopersici*), southern blight (*Sclerotium rolfsii*), Verticillium wilt (*Verticillium dahliae*) and white mold (*Sclerotinia sclerotium*). Among the many viruses affecting chili peppers are alfalfa mosaic alfamovirus (AMV), cucumber mosaic cucumovirus (CMV), beet western yellows luteovirus (BWYV), pepper mottle potyvirus (PepMoV), pepper vein mottle potyvirus (PepVMoV), potato potyvirus Y (PVY), tobacco etch potyvirus (TEV), pepper mild mottle tobamovirus (PepMMoV), pepper ringspot tobamovirus (PepRSV), tomato spotted wilt tospovirus (TSWV), pepper golden mosaic bigeminivirus (PepGMV), pepper Huasteco bigeminivirus (PHV or PepHV), Texas pepper bigeminivirus (TPV) and beet curly top hybrigeminivirus (BCTV).

Production is affected by many insect pests such as cutworms, grubs (*Phyllophaga* spp.), flea beetles (*Eptirix* spp.), hornworms (*Manduca sexta* and *M. quinquemaculata*), grasshoppers, leafminers, fruit worms (*Heliothis assulta* and *H. zea*, *Spodoptera* spp. armyworms, etc.), European corn borer (*Ostrinia nubilalis*), green peach aphid (*Myzus persicae*), melon or cotton aphid (*Aphis gossypii*), leafhoppers, stink bugs, tarnished plant bug (*Lygus lineolaris*), thrips, whiteflies, chili weevil (*Anthonomus eugenii*) and chili pepper maggot (*Zonosemata electa*), and by spider mites (*Tetranychus* spp.) and nematodes.

Chili pepper production is also influenced by physiological disorders such as flower-bud abscission and flower abscission, blossom-end rot, sunscald, abnormal fruit shape, colour spotting, and fruit cracking.

#### D. Experience and world statistics

Chili pepper is harvested at different fruit stages, depending on the final use. Fresh chili pods often are harvested at a physiologically immature (but horticulturally mature) stage. The dehydrated and mash industries use physiologically mature fruits, generally showing red colour.

Chili pungency is measured by determining the capsaicinoids content of the fruit, which can be accomplished by several industrial (laboratory) procedures, and as well by a subjective dilution-and-detection test ("taste test") scored as Scoville Heat Units (Scoville 1912; Korel *et al.* 2002; Bosland and Votava 2000; Krishna De 2003; Reilly *et al.* 2001). Physiologically, capsaicinoids cause the heat sensation by activating and then desensitising certain sensory nerve fibres, which is mediated via a receptor (VR1) in the pain pathway (Caterina and Julius 2001; Bhawe *et al.* 2002). Culinary or medicinal results can be favourable (Rozin 1990; Palevitch and Craker 1995), whereas exposure to excessive amounts can range from avoidance behaviour to severe toxicity (Krenzelok and Provost 1995).

The production of chili pepper for spice, vegetable, and other uses increases every year. It is estimated that it is annually cultivated on more than 1.5 million hectares, in numerous countries (FAO

2001). Forty-six percent of production is in Asia (with China the principal producing country). Southern Europe is the second most important producing region, with 24% of world production. The countries with harvest area of more than 70,000 ha are China, India, Indonesia, Mexico, Korea, Nigeria, Ghana and Turkey.

## 7. Modern Biotechnology

Modern biotechnology can provide benefit to the chili pepper crop by genetic improvement against diseases and insect pests; for enhanced chemical composition, such as in carotenoids and capsaicinoids; and for improved marketing (Bosland and Votava 2000; Ochoa-Alejo and Ramírez-Malagón 2001). Development of a genetically transformed plant requires two key systems: the genetic transformation itself, *i.e.* transferring gene(s) of interest into host cells; and plant regeneration from the host cells with the inserted gene(s). Some other species in the family Solanaceae, such as tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*), have been used as model systems because of their successful transformation and regeneration. Chili pepper however has been recalcitrant, with application of molecular biotechnology lagging because there was not a reproducible, reliable and efficient system of transformation and regeneration (Ochoa-Alejo and Ramírez-Malagón 2001), but these problems are now being overcome.

A few examples of efforts in the biotechnological development of *Capsicum* follow. Exposure to gamma radiation reduced the efficiency of chili pepper shoot regeneration (Sripichit *et al.* 1988). Streptomycin-resistant shoots and whole plants from cotyledon explants were achieved by a regeneration system and chemical mutagenesis [with ethylmethane sulfonate (EMS)] (Subhash *et al.* 1996). A high frequency of plastid-encoded antibiotic-resistant variants were isolated by Rao *et al.* (1997) from seeds and explants mutagenised with EMS or nitrosomethylurea. Dabauza and Peña (2001) improved the efficiency of organogenesis from seedling explants.

The first genetic transformation in chili pepper using modern molecular biotechnology was insertion of the genes for neomycin phosphotransferase and  $\beta$ -glucuronidase by means of *Agrobacterium tumefaciens* (Liu *et al.* 1990); however, these transformed cells did not regenerate into whole plants. Since then, developments in technique for *C. annuum* have been reported steadily, for example, a stable system of *Agrobacterium*-mediated transformation and *in vitro* plant regeneration (Lee *et al.* 1993), a refined protocol for transformation and regeneration (Manoharan *et al.* 1998), a system for highly efficient transformation (40.8%) along with efficient regeneration (Li *et al.* 2003), and the advances and refinements are continuing (Lee *et al.*, 2004b; Mihálka *et al.*, 2003).

Traits currently targeted for development of chili pepper include viral resistance to CMV, TEV and TMV (Cai *et al.* 2003), pest resistance against oriental tobacco budworm (*Heliothis assulta*) (Kim *et al.* 2002), altered fruit ripening, and prolonged shelf life. Diminishing cucumber mosaic virus disease has become a reality, after having developed the fertile transgenic plants with CMV resistance (Zhu *et al.* 1996; Kim *et al.*, 1997). Genetically transformed *Capsicum annuum* with CMV resistance has been approved for commercialisation in China (Huang *et al.* 2002).

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*Part 2*

**CONSENSUS DOCUMENTS ON TRAITS**

*SECTION 1*  
**GENERAL INFORMATION CONCERNING THE BIOSAFETY OF CROP PLANTS MADE  
VIRUS RESISTANT THROUGH COAT PROTEIN GENE-MEDIATED PROTECTION**

**Summary Note**

This document, developed under the auspices of the OECD's Expert Group on Harmonization of Regulatory Oversight in Biotechnology, is intended to provide information that may be of assistance to regulatory officials, developers of virus resistant plants, and other interested parties. Any decision on the use of virus resistant plants at the small- or large-scale stages of product development, or their commercial use, will require a case-by-case review by each Member country, as the specific environment in which such plants will be grown is a component of each of the issues addressed in this document.

The focus of this report is limited to issues that can be discussed in a general fashion without reference to the specific environment in which the transgenic plant is to be introduced. Therefore, any issues relating to the cultivation of the virus resistant plants or to the potential for, or potential effects of, gene transfer from a virus resistant plant to another crop plant or to a wild relative are outside the scope of this document. This document is not intended as an encyclopedic review of all the scientific experimentation on the use of genes to make plant species virus resistant.

The Expert Group identified three topics to be considered in this document as they relate to the use of one specific gene, the viral coat protein, whose expression in plants often results in a resistant phenotype. Two of these topics, transcapsidation and recombination, are known molecular mechanisms through which new virus types may arise; the third topic is the potential for specific synergistic effects that modify symptom development. These are biologically complex phenomena that may sometimes involve at least four different organisms: two viruses, an organism (most often an arthropod) that transmits the virus from plant to plant, and one or more host plant species. These phenomena are not understood in complete detail, and there is considerable research ongoing to address less well-understood aspects.

In some instances, the discussions in this document focus on particular taxa of plant viruses, either those for which the most information is available or those for which the risk issues can be most clearly identified. In addition, the document provides guidance on the biological and molecular information needed to characterise the virus from which the coat protein gene was isolated and the gene inserted into the transgenic plant. Also provided is a list of references that may be helpful in locating such information.

Further research on the basic biology of plant viruses may speed the development of genes for use in virus resistant plants that minimise the potential agronomic or environmental concerns associated with their use, and potentially reduce the likelihood that viral strains will arise that overcome the resistance trait.

## **1. General Introduction**

The following document, developed by a Task Group under the auspices of the Expert Group on Harmonization of Regulatory Oversight in Biotechnology, is intended to be one in a series of documents of use in providing information to regulatory officials, developers of new products produced through biotechnology, and other interested parties.

This document is not intended as a definitive or encyclopaedic review of all the scientific experimentation pertaining to the use of viral coat protein genes to make plant species virus resistant, nor is it intended to dictate to regulatory authorities in any country how they should review requests for field testing, deregulation, or commercialisation of such plants. (For other information on virus resistant transgenic plants, see Hull, 1990, 1994; de Zoeten, 1991; Mansky and Hill, 1993; Falk and Bruening, 1994; Palukaitis, 1991; Tepfer, 1993, 1995; Wilson, 1993.) Rather, the document attempts to describe the current state of experience in Member countries with a particular set of issues pertaining to crop plants made virus resistant through coat protein gene-mediated protection. It draws upon a wide range of information sources, including not only the scientific literature but also risk assessments from Member countries and reports from national conferences and scientific meetings. In an effort to capture the current "state of the art", it also contains preliminary information that may not yet have received full and critical evaluation by the scientific community. Where such information appears, it is indicated as "preliminary".

The issues discussed in this document are a subset of issues that regulatory officials may consider in relation to crop plants made virus resistant through coat protein gene-mediated protection. The focus is limited to issues that can be discussed without reference to the specific environment in which the organism is to be introduced. Therefore, any issues relating to the cultivation of virus resistant plants or to the potential for, or potential effects of, gene transfer from a virus resistant plant to another crop plant or to a wild relative are outside the scope of this document, although these issues are valid considerations that may enter into regulatory deliberations by regulatory authorities in any country. Neither does this document address potential agronomic impacts in any Member country or any other issues that may relate to the potential international marketing of such crop plants.

This document focuses instead on the potential for effects of such genetically modified plants on some natural virus populations or on the severity of viral infections. Specifically, the Expert Group identified three topics to be considered in this document as they relate to the use of one specific viral gene, the gene encoding the viral coat protein, to confer virus resistance. Two of these topics, transcapsidation and recombination, are known molecular mechanisms through which new virus types may arise; the third topic is the potential for specific synergistic effects of particular introduced viral genes on infections with other viruses. These are biologically complex phenomena which may sometimes involve at least four different organisms: two viruses, a viral vector, and a host plant species. These phenomena are not understood in complete detail for all viruses, and there is considerable research ongoing in Member countries to address some of their less well-understood aspects. In some instances, the discussions in this document focus on particular taxa of plant viruses either for which the most information is known, or for which the risk issues can be most clearly identified.

This document represents a consensus of Member countries' positions on factors relevant to addressing the biosafety concerns raised in considering the three identified molecular mechanisms affecting viral populations and viral diseases of plants, as they relate to virus resistant crop plants mediated by CP genes. Current scientific information on these subjects may be sufficient to enable the conduct of case-specific, scientifically sound risk assessments and biosafety evaluations of currently developed varieties. This may enable competent authorities or regulatory officials in countries, after such reviews, to give authorisation for release or commercialisation of particular varieties. The document does not attempt to provide detailed, definitive, or general conclusions on the outcomes of such considerations, nor does it attempt to advise countries on how any such deliberations should be concluded. Member countries have agreed that such deliberations are conducted on a case-by-case basis. Results of particular evaluations of certain issues, as they relate to individual virus resistant crops, are presented for illustrative purposes.

## 2. Technical Introduction to Plant Viruses

Viral diseases cause significant economic losses to agriculture. Viral infections cause damage to fruits, leaves, seeds, flowers, stems, and roots of many important crop species. Under natural conditions, certain plant viruses are nearly always present on particular crop(s) or weed host(s). The types of symptoms produced in a specific plant vary depending on the virus, the specific strain of the virus, whether the plant is infected with another virus or other viruses, the cultivar of the host plant, and the environment. The severity of infection by a particular virus often varies from location to location and from one growing season to the next, reflecting the importance of the environment on symptom development and vector transmission rates for the virus. Some virus outbreaks have been sufficiently severe that entire plantings of target crops (e.g. sugar beet, citrus, and rice) have been destroyed in specific areas. Most crop species are routinely infected with several different viruses. The American Phytopathology Society's Compendium of Plant Disease series lists the important viruses affecting the major crops of the world. *Viruses of Tropical Plants* by Brunt *et al.* (1990) is a useful resource for viral disease of tropical plants.

Plant viruses may be spread in various ways, depending on virus type. Means of spread include: vector-mediated transmission, seed or pollen transmission, and mechanical transmission (whether by transfer of plant sap or by vegetative propagation of infected host tissue). Virus vectors may be nematodes, mites, fungi, or insects. In some environments and for certain viral diseases, substantial inputs of pesticides are needed to control particular vector organisms (typically insects) for serious viral diseases, even though the insects do not themselves cause significant damage to the target crop or could possibly be controlled by biological means. Control of the vector organism does not always result in complete or effective control of the viral disease. In addition, in certain environments particular crop species cannot be grown profitably because of the presence of persistent populations of infected plants and potential vectors. The situation with soil-borne (nematode or fungus transmitted) viruses is even more dire. If these infested vectors become established at a site, eradication or even satisfactory control is usually impossible or environmentally untenable. Unless resistant cultivars are available, cultivation of the susceptible crop at that site may have to be abandoned. Examples include infections of Indian peanut clump furovirus in groundnut in parts of India, and rhizomania disease in sugar beets [caused by beet necrotic yellow vein virus (BNYVV)] in the United Kingdom.

Plant viruses are relatively simple pathogens, in essence protein coats (capsids) wrapped around genomes of either DNA or RNA. Some capsids may also contain carbohydrates and/or lipids. The viral genome encodes at least its own nucleic acid replicating enzyme, (a) protein(s) required for movement of the virus throughout the plant, the viral coat protein(s), and often other necessary proteins. After entering a host plant cell, a virus particle (virion) uncoats, replicates copies of its genome, uses its CP gene to manufacture the protein subunits for the virus protein coat, and then assembles new virions. The new virions or infectious agents may spread to adjacent cells or be transported by vector organisms to other host plants.

Plant viruses are usually named according to the plant species in which they were first detected and the type of symptoms observed in infected plants. The genome of each plant virus is of a characteristic composition, DNA or RNA, either single-stranded or double-stranded depending on the virus. Some plant viruses contain more than one nucleic acid molecule within each virus particle. For other viruses, the genomes consist of more than one nucleic acid molecule, each packaged in a separate virion. Some viral infections are also associated with the production of satellite RNAs or satellite viruses. Satellite RNAs depend on a specific virus (called helper virus) for the replication enzymes needed to replicate their own RNA, are usually smaller in size than their helper viral genome, have no significant sequence homology to the helper virus genome, and affect disease symptoms (at least in some hosts) (Matthews, 1991). In satellite viruses, the satellite codes for its own coat protein, while satellite RNAs are packaged in the coat protein of the helper virus. Plant viruses are taxonomically grouped according to their nucleic acid

composition and other physical properties of the virions. Nucleic acid sequencing of hundreds of animal and plant viral genomes has revealed the evolutionary relationships among many viruses. An important reference for viral taxonomy is Murphy *et al.* (1995), a publication of the International Committee on Taxonomy of Viruses (ICTV).

Plant viruses have traditionally been controlled in agriculture using a variety of strategies with varying effectiveness: exclusion of contaminated material at national or state borders with accompanying virus identification (often by indexing); roguing of infected crops; plant eradication measures, when appropriate; certification of virus-free stock or seeds (*e.g.* to control plum pox potyvirus in fruit trees and for many viruses in potatoes); use of agronomic practices designed to minimise virus spread or persistence (*e.g.* not planting a particular crop for a specified period of time in a given locality); conventional breeding for virus-resistant cultivars; and conventional cross-protection (*i.e.* pre-inoculation of plants with a mild strain of the virus to protect against severe infection by another strain of the same virus) (used to varying degrees to control tomato mosaic tobamovirus in tomatoes in Europe and Japan and citrus tristeza closterovirus in Brazil). Conventional cross-protection, while commercially important for some crops in certain localities, is only effective for certain viruses. It involves intentional infection of crop plants with a suitable closely related mild virus strain, provided that such a virus strain is available. Two of the main issues associated with the development of useful conventionally-bred, virus resistant crop lines have been 1) identification of breeding stock containing an appropriate resistant trait/gene(s); and 2) potential trade-offs between introgression of the virus resistant trait and other agronomically important traits in the crop itself.

Until 1994, when Whitman *et al.* cloned and sequenced the tobacco N gene conferring resistance to TMV, no plant-derived traditional viral resistance gene had been cloned or sequenced. The exact function of the N gene is still not understood, although recent evidence suggests its involvement in a common signal transduction mechanism for general pathogen resistance (Staskawicz *et al.*, 1995). Nonetheless, introduction of traditional resistance genes into agronomically desirable cultivars has been used for decades to protect plants from viral infections even though their mode of action has not been understood. The lack of detailed understanding of the mechanism of traditional resistance genes, or traditional cross protection measures, has not prevented their use.

Another type of strategy for protecting a plant against viral disease involves introduction and expression of a gene encoding the viral CP in the genome of the plant itself. This type of strategy is referred to as "coat protein gene-mediated protection", and its effectiveness was first demonstrated on tobacco mosaic tobamovirus infection of tobacco in 1986 by Powell Abel *et al.* It provides heritable protection of the recipient plant species against the target virus, and frequently against related strains as well. This strategy has been demonstrated in laboratory or field experiments to be effective against at least 50 different viruses to date. Since that time, viral genes other than CP genes [dedicated movement proteins, replicase (polymerase), viral genes modified to contain ribozymes, satellite and defective interfering RNAs] have also been shown to confer a virus resistant phenotype on recipient plants. The growing number of genes used to encode virus resistance is more illustrative of the diversity of the viruses against which resistance is targeted than the plant species they infect. However, this document focuses exclusively on the biosafety of those genetically modified plants made virus resistant through the introduction of a viral CP gene, and on biosafety with respect to interactions of the modified recipient plant with other plant viruses in the environment.

### 3. Basic Information for Virus Characterisation

The information relevant for a biosafety review of an organism includes that which establishes the identity of the organism in question and that which describes the environments in which the organism is to be used. Any genetically modified plant protected against viral infection through CP gene-mediated

protection will potentially interact with the range of organisms with which the parental plant species can interact within the same environment. Characterisation of the virus which provided the transgene would include information on virus biology, taxonomy, genetics, and known viral interactions in the environment. This necessary information would include:

- a. The taxonomic name of the virus, including family, genus, and strain designation, including any synonyms.
- b. The type of nucleic acid contained in the virus.
- c. Whether the infection is systemic or localised.
- d. Whether the virus is restricted to specific tissues (*e.g.* phloem-limited).
- e. Whether the virus is associated with any satellite or helper viruses.
- f. The natural host range of the virus.
- g. How the virus is transmitted.
- h. If the virus transmitted by a vector, the identity of the vector including mode of transmission (*e.g.* persistent or non-persistent) and the identity of the viral gene(s) (if known) involved in vector transmission.
- i. Whether any synergistic or transcapsidation interactions with other viruses under field situations have been reported in the literature.

In order to evaluate any potential biosafety concerns posed by the use of viral genes, viral sequences engineered into the plant should be well-characterised sequences that are derived from well-characterised viruses, and the specific biological properties of the actual strain utilised should be known. Characterisation of the strain from which the transgene is derived may enable determination of whether that strain is identical or nearly identical to the strain found in other countries. For example, beet necrotic yellow vein furovirus strain A that is widely prevalent in Japan, the United Kingdom, the Netherlands, and other parts of Europe is virtually identical to the strain found in the United States (Kruse *et al.*, 1994). A considerable amount of data on viral strains is readily available in scientific publications and from publicly accessible data bases.

For appropriate designations of most plant viruses, the official taxonomic body for virology is the International Committee on Taxonomy of Viruses (ICTV), which has published the accepted taxonomic names for most plant viruses (Murphy *et al.*, 1995). Relevant types of information to describe the virus in question are the complete name of the virus (including any synonyms), the family and genus names, the strain designation, the name of the disease incited, and the locality where the strain was first isolated. The molecular characteristics of a plant virus most important for describing the properties of viral infections are the type of nucleic acids contained, RNA or DNA, and whether those nucleic acids are single- or double-stranded (Murphy *et al.*, 1995). It is important to describe whether the virus replicates in all cells (*e.g.* tobamoviruses) or is limited to certain cells (*e.g.* phloem cells for luteoviruses).

Although an up-to-date, definitive, and concise list of the host ranges of all plant viruses is not available, several publications and Internet sites have a significant amount of useful information:

- a. The Commonwealth Mycological Institute/Association of Applied Biologists' "Description of Plant Viruses" is a series of pamphlets describing the biology of several hundred plant viruses.
- b. The USDA's *Plant Pests of Importance to North American Agriculture, Index of Plant Virus Disease* (Agriculture Handbook No. 307, 1966) provides a list of plants and the viruses that infect them.

- c. The American Phytopathology Society's series on plant diseases of crops has up-to-date listings of viral diseases for the major crops. The Society also has a list of names of U.S. plant diseases and their causal agents.
- d. The Australian Virus Identification Data Exchange (VIDE) is currently being promoted by the ICTV to establish a worldwide database dealing with plant viruses. The World Wide Web site for the database is: <http://life.anu.edu.au/viruses/ictv/index.html>.
- e. The British Society for Plant Pathology's *Names of British Plant Diseases and their Causes*, published in 1984, lists the English and European names of the diseases and the scientific names of the causal organisms, arranged by host plant.
- f. The *European Handbook of Plant Diseases* by Smith *et al.* (1988) provides descriptions of the viral, bacterial, and fungal pathogens of European plants.
- g. A World Wide Web site maintained by the Garry Laboratory at Tulane University (USA) has as its goal to provide a list of all virology sites on the Word Wide Web. This site can be accessed at <http://www.tulane.edu/~dmsander/garryfavweb.html>. A mirror site has been established to facilitate access in Europe at the University of Leicester (United Kingdom) (<http://www-micro.msb.le.ac.uk/335/garryfavweb.html>).

Many publications describe the host range of a particular virus. However, most lists do not describe the host range of specific viral strains. Host range is an important consideration for the three issues with which this document is concerned. Because of the number of different strains of a particular virus, information on the natural host range of the specific viral strain used as a donor organism may be more easily provided by the person who has engineered that plant than by a literature search. Information on the natural host range of a viral strain in managed and unmanaged ecosystems is probably more relevant than information on its "artificial" host range. The natural host range of a virus lists the plants growing in managed and unmanaged ecosystems that are commonly infected with the virus. The artificial host range includes plants that become infected when intentionally inoculated by man under controlled conditions but are not necessarily infected under natural conditions. The artificial host range of a virus includes more plant species than the natural host range (Matthews, 1991).

A definitive worldwide list of the geographical distribution of plant viruses is also unavailable. However, limited information on the geographical distribution of many plant viruses can be found in the references listed above. The United States Department of Agriculture (USDA) has a state-by-state list of occurrences of widely prevalent viruses on its World Wide Web Site (<http://www.usda.gov/bbep/bp>).

Viruses are transmitted by many vectors, including whiteflies, mites, nematodes, aphids, planthoppers, leafhoppers, beetles, thrips, and fungi. They can also be transmitted mechanically and through seed or pollen. For those viruses that are vector-transmitted, a single virus is transmitted under field conditions by a single vector group. Thus, as an example, three viruses from three different genera of the family Potyviridae, potato Y potyvirus (PVY) (genus *Potyvirus*), ryegrass mosaic virus (genus *Rymovirus*), and barley yellow mosaic virus (genus *Bymovirus*) are transmitted by unrelated types of vectors. In this example several aphid species transmit the first virus, the mite *Aceria tulipae* transmits the second, and the fungus *Polymyxa graminis* transmits the third virus. Each group of vectors transmits particular viruses worldwide (Murphy *et al.*, 1995).

Although these three viruses are all in the same family, they are transmitted by only one specific type of vector. The high specificity of this virus-vector relationship is a result of interaction between specific vector-encoded receptors and the specific virus-encoded protein(s) that is unique to each virus (Murphy *et al.*, 1995; Murant *et al.*, 1988b). Identifying the major vectors of field importance (both scientific and common names) is part of the characterisation of both the virus itself and the recipient environments. In addition, if any viral genes have been identified as being implicated as required for vector transmission, the

nature of the genes and, briefly, how they are believed to be involved in vector transmission should be described.

#### 4. Expression of Coat Protein Gene in Transgenic Plants Results in a Virus Resistant Phenotype

Powell Abel *et al.* (1986) showed that transgenic plants expressing the CP of tobacco mosaic tobamovirus (TMV) imparted resistance to TMV. Since that time, over 30 plants, both monocots and dicots, have been engineered to express more than 50 viral CP genes from ten taxa. Many of these have been field tested. One of the catalysts for this research has been knowledge of the phenomenon of cross protection, in which a plant infected with a mild strain of a virus is often protected from infection by a severe strain of the same virus. Although the exact mechanism by which cross protection works is not clear, evidence suggests that CP is involved with some viruses (Matthews, 1991).

Cross protection has been used in agriculture for many decades worldwide. Currently in Japan, more than half a million tomato plants (for both fresh market and processing uses) are cross protected against cucumber mosaic cucumovirus (CMV) (containing a satellite RNA of Japanese origin) (Sayama *et al.*, 1993; Sayama, unpublished data). Tomatoes cross protected with tomato mosaic tobamovirus have been or are being consumed in Europe and Japan (classically-bred resistant cultivars are also widely used), citrus trees have been protected against citrus tristeza closterovirus in Brazil (Fulton, 1986), papaya trees have been protected with papaya ringspot potyvirus (as reviewed by Yeh *et al.*, 1988), and zucchini plants have been protected with zucchini yellow mosaic potyvirus. Before indexing was widely used for virus elimination in potato, many potato seed pieces were infected with mild strains of many common viruses, including potato leaf roll luteovirus, potato X potexvirus, and potato Y potyvirus (Hooker, 1981), and therefore were cross protected using traditional techniques. These methods are still used for many vegetatively propagated plants, like strawberries, as well as florist and nursery crops.

Coat protein gene-mediated protection is best understood with TMV and tobacco. A brief summary of the current state of knowledge of this system is summarised below. For protection of tobacco to be effective, TMV CP must accumulate. Development of protection does not seem to involve the induction of the plant's natural disease resistance system. Resistance appears mainly to be based on blocking the uncoating of the CP in the incoming TMV. There is, however, evidence that a later step in infection is also affected (Reimann-Phillip and Beachy, 1993). It has been observed that protection is better when the CP is derived from a viral strain that naturally infects the recipient plant than when the CP is derived from a closely related strain that infects another host plant. Tomato plants expressing tomato mosaic tobamovirus (ToMV) CP gene, the tobamovirus most closely related to TMV, are better protected from ToMV infection in the field than tomato plants expressing tobacco mosaic tobamovirus CP (Sanders *et al.*, 1992). Resistance derived from the CP gene of other viruses may have modes of action different from TMV.

Based on the success of CP gene-mediated protection during field testing, most plant virologists believe that CP gene-mediated resistance may be successfully applied for many but not all (Ploeg *et al.*, 1993) single-stranded, positive sense RNA viruses, a group which includes over 75 per cent of all plant viruses (Beachy, 1993). More field tests of virus resistant plants have occurred in the U.S. than in any other OECD Member country. In the U.S., most but not all of the CP genes have been derived from viruses that commonly infect the recipient crop. A majority of the viral sequences that have been introduced into transgenic plants and field tested thus far have not been modified from the original sequence found in the parental virus, except for modifications related to cloning of the gene. A few CP genes have been modified so that the ability of the virus to be transmitted by its vector is significantly reduced; others have been isolated from strains that were non-transmissible by the vector under natural conditions. In some cases, expression of CP gene from a viral strain that does not naturally infect the plant can provide resistance to taxonomically related virus that may or may not naturally infect the plant (Stark and Beachy, 1989; Namba *et al.*, 1992).

Another approach involves using a CP gene that has been modified by removing some of the nucleotide sequences from the gene, resulting in a truncated CP (Lindbo and Dougherty, 1992 a,b). Depending on how much of the gene is deleted, the CP derived from the truncated transgene may or may not be able to function in virion assembly (Lindbo *et al.*, 1993). Dougherty's laboratory (Smith *et al.*, 1994; Lindbo and Dougherty, 1992 a,b) has shown that a modified CP transgene that encodes a non-translatable mRNA may also provide protection. This resistance may result from direct interaction of transgene RNA and viral RNA, commonly referred to as RNA-mediated resistance, although host factors may also play a role in resistance (Smith *et al.*, 1995).

Antisense expression (the production of complementary, non-coding transcript of a gene) of coat protein gene has generally not been as effective as sense expression in protecting plants against viral infections, although there are some notable exceptions (Hammond and Kamo, 1995; Kawchuk *et al.*, 1991; Lindbo and Dougherty, 1992 a,b). This low success rate as compared to sense expression may not be unexpected, since antisense strategies act at the level of gene expression in the nucleus whereas most plant viruses multiply in the cytoplasm (Beachy, 1993). Whether antisense-, truncated sense-, or untranslatable sense-mediated resistance is as effective in providing immunity or resistance as sense CP-gene-mediated protection under field conditions needs further investigation. If the CP-derived transgene produces a CP which cannot encapsidate viral nucleic acid or does not produce a CP, this minimises the issues addressed in Section V (transcapsidation and synergy).

CP-gene mediated resistance will probably not be totally effective against virus strains that have satellite RNAs associated with them. These small RNAs can often modify the symptoms expressed by infected plants. Depending on the genotype of the host plant, the sequence of the small RNA, the helper virus, and environmental conditions, the symptoms may be attenuated or more severe (Matthews, 1991). Although satellites and defective-interfering RNAs have been detected in some viruses, their role in disease development under natural conditions is unclear. For the majority of viruses for which satellite RNAs have been detected, satellites are rarely found in virus-infected plants in the field, nor have they ever been shown to have caused a severe epidemic. There are two major exceptions. One is that of the satellites of CMV, which have caused serious disease epidemics in China, Italy, Japan and Spain in the past decade (Tien and Wu, 1991; Kaper *et al.*, 1990). The other is groundnut rosette virus, of which all the isolates that cause rosette symptoms contain satellite RNAs (Murant *et al.*, 1988a). Coat protein gene-mediated protection alone does not protect plants against infection if the virus contains satellite RNAs, so that additional measures are likely to be necessary for engineering effective protection against such satellite-containing viruses (Yie and Tien, 1993).

In the Sixth Report of the International Committee on Taxonomy of Viruses (ICTV), the genus *Umbravirus* was recognised with carrot mottle as the type species (Murphy *et al.*, 1995). Umbraviruses have worldwide distribution, but have been found only in plants co-infected with luteovirus. Umbraviruses can be distinguished from luteoviruses based on the fact that Umbraviruses are manually transmissible, whereas luteoviruses are only aphid-transmitted. However, in the field Umbraviruses are genomically masked by luteoviral coat protein and thus aphid-transmitted. On the basis of biological properties, four Umbraspecies have been recognised by the ICTV and four additional candidate species have been proposed. No reports have been published regarding transgenic plants engineered to be resistant to Umbraviruses, and the luteovirus resistant plant likely to be commercialised within the next few years (see Section V) contains a non-capsid gene as the source of the resistant phenotype. For further information, see the papers cited in the second paragraph of Section I.

## 5. Issues Related to Potential Effects of CP Gene-mediated Virus Resistance on Virus Infections

Although more than 50 virus resistant plants using CP gene-mediated resistance have been field tested worldwide to date, it is likely that only a limited number of these will be commercially available in the

next few years. Some of the virus resistant plants that may be eligible to be considered for commercialisation in the next few years in OECD Member countries might be:

- beet necrotic yellow vein furovirus resistant sugar beets;
- tomato mosaic tobamovirus resistant tomatoes;
- potato leaf roll luteovirus resistant potatoes; \*
- potato X potexvirus resistant potatoes;
- cucumber mosaic cucumovirus resistant tomatoes, peppers and cucurbits;
- zucchini yellow mosaic potyvirus, watermelon mosaic potyvirus 2, and papaya ringspot potyvirus resistant cucurbits;
- potato Y potyvirus resistant potatoes;
- potato Y potyvirus resistant tobacco;
- cucumber mosaic cucumovirus resistant tobacco; and
- papaya ringspot potyvirus resistant papayas.

\*In North America, Europe and Japan the PLRV resistant lines likely to be commercialised use a non-CP gene as source of the resistance phenotype. PLRV CP-mediated resistance has also been field tested in many countries.

Some plants that may be commercialised could contain combinations of the above resistance genes. An attempt is made in this document to highlight information or data which may be particularly relevant to the above listed viruses.

Three distinct interactions, transcapsidation, synergy, and recombination, have been observed to occur when two plant viruses (or two different strains of the same plant virus) simultaneously infect a cell. A brief description of each of these interactions is provided, followed by an analysis of how each may play a role when transgenic plants are made virus resistant through the use of CP gene-mediated resistance.

#### A. Transcapsidation

When a single plant cell is simultaneously infected by two different strains of a virus (or two viruses), it may be possible for the genome of one virus to become encapsidated by coat protein of the second virus. If the virus is encapsidated by coat proteins of both viral strains, the phenomenon is called phenotypic mixing (mixed encapsidation). If the virus is encapsidated by only one of the coat proteins, this is termed genomic masking or transcapsidation. (For simplicity, it will be assumed that the terms transcapsidation and genomic masking include the phenotypic mixing phenomenon, since the issues for all are identical). Transcapsidation has been reported to be important in only a few instances in field situations in insect transmission of viruses (Falk *et al.*, 1995), even though field grown plants and trees are known to be infected with multiple viruses (Abdalla *et al.*, 1985; Falk and Bruening, 1994).

Transcapsidation has been best studied with infections with different strains of the barley yellow dwarf luteovirus, where the phenomenon can be important in field situations in that coat protein determines which specific aphid vector transmits the virus (Matthews, 1991). This phenomenon has also been detected with potyviruses (Bourdin and Lecoq, 1991; Lecoq *et al.*, 1993) and tombusviruses (Dalmay *et al.*, 1992). (Similar preliminary results have also been reported with nepoviruses (Hiriart, 1995). The result of transcapsidation, a "masked" virion, has a mismatched coat that may or may not be sufficiently functional to allow transmission of the viral genome it contains to another host plant. The "mismatched" or heterologous viral coat is not maintained in subsequent rounds of viral infection, because subsequent production of coat protein subunits is directed by the viral coat protein gene carried in the genome.

Therefore, transcapsidation events are transient and any potential impacts can only persist with the first round of infection of the masked virus if it infects a susceptible host plant.

For some viral taxa, a protein other than CP is the primary determinant of whether a specific organism can successfully transmit a virus. These taxa include potyviruses, caulimoviruses, and waikaviruses (Murphy *et al.* 1995). This vector transmission protein is called a "helper component" in potyviruses and an "aphid helper transmission factor" in caulimoviruses (Murphy *et al.*, 1995). Unless the appropriate vector transmission protein is present and functional, transcapsidated virions assembled with CP from a vector transmissible strain will not be efficiently transmitted by the "heterologous" insect vector (Berger *et al.*, 1989; Atreya *et al.*, 1990). In contrast, viral CPs apparently are the primary determinants for insect-transmissibility for geminiviruses and cucumoviruses (Matthews, 1991). In the fungus-transmitted furoviruses and the aphid-transmitted luteoviruses, the vector transmission protein is synthesised by read-through of the CP termination codon (Zaccomer *et al.*, 1995; Schmitt *et al.*, 1992; Wang *et al.*, 1995). Rice tungro waikavirus is required for aphid-transmission of rice tungro bacilliform badnavirus, and thus probably encodes an aphid-transmission protein (Dasgupta *et al.*, 1991). For some taxa, little is yet known about the nature of the protein(s) involved in vector transmission.

Two issues are important to be addressed in considering the likelihood and significance in any potential instance of transcapsidation in transgenic plants. As stated above, if a resistant plant was engineered with a gene that does not produce a CP, or produces one that cannot function in the assembly of virions, these issues need not be addressed:

1. Is there a sufficient amount of coat protein being produced by the transgenic plant to produce a masked virus? Is the CP found in the same or different tissue(s) where the virus is detected in a non-transgenic plant?
2. If a masked virus were produced, would it have any new biological properties (vector transmission and host range) and would any effects resulting from transcapsidation be measurable or significant?

As mixed infections by plant viruses of all taxonomic types are common in nature (Zink and Duffus, 1972; Davis and Mizuki, 1987; Duffus, 1963), it is likely that there are many as yet unrecognised examples of heterologous transcapsidation interaction that naturally occur between plant viruses. However, research thus far indicates that heterologous transcapsidation interactions occur only in specific interactions in most mixed infections. There is evidence for both traditional and transgenic virus resistant plants that transcapsidation may occur (Rochow, 1972; Matthews, 1991; Farnelli *et al.*, 1992; Osbourn *et al.*, 1990; Dalmay *et al.* 1992; Holt and Beachy, 1992; Lecoq *et al.*, 1993; Maiss *et al.*, 1994; Candelier and Hull, 1993).

With the impending commercialisation of transgenic virus resistant plants, an important consideration is whether the use of viral CP-expressing transgenic plants might increase the possibility for heterologous transcapsidation interactions to occur and, if the possibility is increased, whether it poses a significant risk. One way in which scientists have sought to assess potential transcapsidation frequency in transgenic virus resistant plants has been to compare the amount of the engineered coat protein in the transgenic plants with the amount of coat protein in a similar, but susceptible, non-transgenic plant (Issue 1 above). One hypothesis has been that comparable or smaller amounts of coat protein would lead to the prediction that the transcapsidation frequency will be comparable or reduced from the frequency that occurs in naturally occurring mixed infections.

A second consideration would be whether the transgene CP is synthesised in the same tissues that the virus naturally infects in non-transgenic plants. If CP synthesis takes place in the same tissues, then no new

interactions with other viruses that may be limited to other plant tissues can occur. The amount of transgene CP that can be detected in a transgenic plant may increase if the plant is infected by a related virus to which it is susceptible (Farnelli et al., 1992). The increase in detectable CP transgene may be a result of the CP being stabilised in masked virus particles rather than to an increase in transgene mRNA. It may be prudent to ascertain the amount of detectable CP transgene and mRNA in a transgenic plant when inoculated with common viruses with which the transgenic plant would routinely become infected in field situations.

One example of these considerations having entered into the regulatory assessment process in an OECD Member country is the Asgrow Seed Company's ZW20 squash, which is engineered to be resistant to zucchini yellow mosaic potyvirus (ZYMV) and watermelon mosaic potyvirus 2 (WMV2) by the expression of their respective CP genes. The review of ZW20 was conducted by the United States. At the time of preparation of this consensus document, it is the only virus resistant plant that has completed the reviews necessary to allow agricultural use of the plant in an OECD Member country. In ZW20 plants, the review concluded that the CPs are expressed in the same plant tissues in which the corresponding viruses are normally detected, and that the amount of CP produced in ZW20 plants is less than, or equal to, the amount in naturally infected plants. The amount of transgene CP detected increased in ZW20 plants after infection with papaya ringspot potyvirus (PRSV), although transgene RNA concentration did not increase. The amount of transgene CP detected in PRSV-infected ZW20 was still less than that found in PRSV-infected squash plants. In a review of Asgrow's next squash line (CZW-3), which is resistant to cucumber mosaic cucumovirus, ZYMV, and WMV 2, no increase in transgene CPs was detected when the transgenic plants were challenged with PRSV.

It has been demonstrated that heterologous transcapsidation can occur in transgenic plants that express viral CP (Osbourne et al., 1990; Dalmay et al., 1992; Holt and Beachy, 1992). Lecoq et al., 1993 showed that when plants expressing a CP transgene derived from an aphid-transmissible strain were challenged with a non-aphid transmissible strain (defective in CP not aphid transmission factor), a heterologous aphid transmissible strain was detected. Another important question is whether transcapsidation can occur with more distantly related viruses. Candelier-Harvey and Hull (1993) have shown that when plants expressing the CP of alfalfa mosaic alfalmovirus (AIMV) are infected with cucumber mosaic cucumovirus (both members of the family Bromoviridae), the CMV genome is encapsidated in particles that contain AIMV CP. Since AIMV has no known insect vector, it was not possible to evaluate changes in vector specificity. It seems that if heterologous transcapsidation occurs in these plants as a result of virus infection in the field, there are at least two biologically significant outcomes to be considered. These are: 1) that heterologous transcapsidation events in the transgenic plants could alter or facilitate vector transmissibility of the new progeny virions (those generated as a result of heterologous transcapsidation); and 2) that heterologous transcapsidation events in transgenic plants could facilitate systemic movement of the resulting progeny virions within the transgenic plants when they belong to a plant species in which the "normal" virus (that not resulting from heterologous transcapsidation interactions) does not readily move systematically. If the first scenario were to occur, and a virus were to gain vector transmissibility via heterologous transcapsidation with the transgenic plant, would the potential for new disease development be great either within the transgenic crop or in other plants? It is impossible to predict the answer for all situations, because cropping situations, geographic location, type of vector and its abundance, local crops and other factors will vary greatly from one country to another (Falk et al., 1995). Each of these scenarios will be discussed.

**Scenario 1A. Altered vector transmission and disease development in the transgenic crop.** If vector transmission of a plant virus were altered or facilitated as a result of heterologous transcapsidation interactions resulting from infection of a CP-expressing transgenic plant, it is not known whether this would cause significantly greater virus spread and disease development with the transgenic crop. In this scenario, any virus spread to a new transgenic plant as a result of heterologous transcapsidation would

contain CPs derived from the CP-expressing transgenic plant. If these masked viruses were subsequently vector-transmitted to another CP-expressing plant within the same field (secondary spread), they might or might not be able to infect such a plant. In one experiment, Osbourn et al. (1990) challenged transgenic tobacco plants expressing the functional coat protein derived from UI strain with a strain (DT1) of TMV that exists only as unencapsidated RNA. (The CP of this strain is defective.) Virions were produced that contained DT1 RNA encapsidated by UI CP. When the masked virions were inoculated onto transgenic tobacco plants expressing UI CP, the plants were resistant to infection. Control non-transgenic plants showed the expected symptoms. This supports the notion that secondary spread of masked virions is unlikely to occur within the transgenic crop, as the plants would be resistant to the masked virions.

Although transcapsidation may be detected under laboratory conditions, field tests under natural conditions will indicate whether the secondary spread of heterologous transcapsidated virions is likely. As part of an ongoing multi-year experiment to determine the potential biological impacts of transcapsidation, Dr. Gonsalves and co-workers have been attempting to determine whether there are biological impacts of transcapsidation in a field situation (Gonsalves et al., 1994; Fuchs and Gonsalves, 1995). Melon, squash, and cucurbit plants were developed that express the CP from a highly aphid-transmissible strain of CMV, strain WL. (The CP is known to be the only determinant in aphid-transmission in cucumoviruses.) Depending on the plant line used, the CP transgene may be expressed at relatively high or low concentrations. In the 1993 and 1994 growing season, these plants were grown in the field and challenge inoculated with a strain of CMV (strain C) that was not aphid-transmissible. The researchers looked in their inoculated transgenic plants and healthy, non-inoculated control plants for transcapsidated aphid-transmitted CMV. This transcapsidated CMV would have contained RNA from strain C, encapsidated with CP from WL strain derived from the plant transgene. Thus far, the spread of CMV C from inoculated transgenic to healthy non-transformed plants has not been detected. [Similar indications are also apparently emerging from the 1995 field trial (Fuchs, unpublished data)]. These experiments have been performed in a locality where the crops are routinely grown, the aphid vectors are abundant, and CMV is a serious problem in these crops. Further studies with other virus-crop systems will be useful in trying to confirm these findings (for scenario 1a) for other virus-plant systems and environmental conditions.

**Scenario 1B. Altered vector transmission and disease development with another plant.** It is also possible that if heterologous transcapsidation were to occur in CP-expressing transgenic plants, the resulting masked virion might be transmitted by the "new" vector to another plant or crop that is not transgenic. In this scenario, the transgenic crop would serve as a new virus reservoir (for the heterologous transcapsidated virus), allowing virus spread to a new plant. However, spread of heterologous transcapsidated virions to the second plant would only be primary spread, *i.e.* spread from the transgenic plants where transcapsidation took place to a different plant species. Once the chimeric transcapsidated virions infect a new, non-transgenic host, they will again resort to the phenotype determined by the viral nucleic acid, as the only capsid protein source in these plants would now be their own genome. For these viruses to spread secondarily through the non-transgenic plant population, they would now have to be spread by their original vector, which may or may not be present (Falk et al., 1995). With respect to the potential effects of any primary spread, for many plant viruses, especially those of annual crops, the most common and economically important form of virus spread is secondary spread (Simons, 1959; Alderz, 1978). Primary spread generally involves few, or a limited number of, plants and in most cases does not result in economically important losses. Secondary spread, in contrast, can be rapid and involves spread from the initial, primary infected plants to the large numbers of remaining healthy plants (Matthews, 1991). Thus, if in the above example the transgenic plants were to serve as sources for primary spread of chimeric transcapsidated virion to another crop, disease and virus incidence from the primary spread would likely be limited in scope. Secondary spread in the non-transgenic plant could only occur if the natural vector(s) of the wildtype virus were already present. However, if the natural vector were already present, then it is possible that the natural vector could provide for primary as well as secondary spread, and both would spread wildtype virus (Falk et al., 1995). Of course this scenario, which deals with epidemiology of

virus spread, depends greatly on the virus, vector, and local-site specific conditions, which might require case-by-case review.

**Scenario 2. Disease development resulting from new systemic spread within transgenic plants.**

The movement of a virus from the initial site of infection throughout a plant, called systemic infection, requires expression of one or more viral genes (a dedicated movement protein, coat protein, and/or viral proteins) and a permissible host plant (Hull, 1989; Maule, 1991; Dawson et al., 1988; Marchoux et al., 1993; Dolja et al., 1995; Cronin et al., 1995; Valkonen and Somersalo, 1995). If a virus is unable to move from the initial site of infection, these infections are called subliminal. In a limited number of cases, viruses that cause subliminal infections in a host species may no longer be restricted when the host is infected by a second virus. In a large number of these studies (Atebekov and Talinsky, 1990) it has not been determined whether the coat protein is solely responsible for this helper dependent movement, but for viral taxa where a dedicated movement protein has not been described, consideration that the coat protein is the primary determinant of movement should be noted. If the coat protein expressed in the transgenic plant can facilitate the movement of viruses that cause subliminal infections, this would be a significant concern only if that CP was from a virus that rarely or never infects the recipient host plant. If CP is derived from a virus that is widely prevalent in the recipient plant, there would be no new novel interactions with subliminally-infecting viruses. This situation is true for the transgenic plants that are likely to be commercialised during the next few years (see Section V). This assumes the transgene is expressed in the same cells as virus. There are several situations in which this type of interaction may need further review.

- a. Although the virus that provided the transgene may be widely prevalent in many countries, different strains may be present in different countries. Whether the biological properties of the transgene CP are identical to those of the CP from the viral strains present in another country would require a review.
- b. If the virus that provided the CP transgene was not present in a country, then there could be new interactions between the transgene CP and viruses that cause subliminal infections. However, it is unlikely, but not inconceivable, that regulatory agencies in a country would be asked to approve a virus resistant plant where the virus was not an economically important pathogen.
- c. If the virus that provided the transgene CP was present in the country, but was usually found in a different plant species from that of the recipient transgenic plant, there could be new interactions between the transgene CP and subliminally-infecting viruses.

In all these cases, if the viruses that cause subliminal infection of the recipient host are known, then easily performed tests can be conducted to determine whether CP facilitates their systemic movement. Whether the movement of the virus in the transgenic plant results in significant disease loss will depend on the virus, plant, and environmental conditions in each locality. Whether the virus can move from the transgenic plant will depend on its mode of transmission, especially whether viral vectors are present and feed on the transgenic crop.

Although not all of the useful experiments regarding the potential effects of heterologous transcapsidation have been completed, reports published in two OECD Member countries have reached certain conclusions about the potential risk concerns posed by heterologous transcapsidation. The conclusions reached in these two countries may not necessarily apply to all Member countries. In its report to the United Kingdom's Ministry of Agriculture, Fisheries, and Food entitled "Risks to the Agricultural Environment Associated with Current Strategies to Develop Virus Tolerant Plants Using Genetic Modification," Henry et al. (1995) state: "The general view is that transcapsidation is not a problem, because it is limited to a single transfer, *i.e.* once a transcapsidated genome is introduced into a new host, it

reverts to using its own CP." In the report of a workshop on virus resistance prepared by the American Institute of Biological Sciences (AIBS) for the U.S. Department of Agriculture, a similar finding is reached (AIBS, 1995): "Transcapsidation of viral RNAs with coat protein produced by transgenic plants should not have long-term effects, since the genome of the infecting virus is not modified."

In conclusion, the potential impacts of transcapsidated viruses from viral CP-gene protected plants is generally expected to be no more serious than the impacts that occur in multiple viral infections of susceptible crops. However, there are a few cases with certain viral taxa where questions remain. Many of these potential impacts can be addressed via currently funded research or during variety development.

## B. Synergy

Occasionally, when two viruses simultaneously naturally infect a plant, the symptoms can be more severe than when either of the viruses infects the plant singly. This phenomenon is called synergy (Matthews, 1991). Synergistic infections can often result in severely diseased, unmarketable crops. Synergy was first described and is best studied with PVX and PVY. The majority of synergistic viral combinations include, as one the viruses, a potyvirus (see Table 2.1, listing some synergistic interactions, which was prepared by Dr. V. Vance, University of South Carolina, U.S.A.). [The discussion here is limited to viral interactions that affect symptom development. Other specific interactions, *e.g.* the ability of TMV to move systemically in barley in the presence of brome mosaic bromovirus (Hamilton and Nichols, 1977), which probably result from movement protein complementation, will not be discussed.]

Will coat protein-mediated resistance produce unintended synergistic symptom expression when the resistant plant is infected by other plant viruses? Since potyvirus CP genes are not involved in synergism, it is unlikely that infection of a transgenic potyvirus resistant plant with any other virus would result in a synergistic interaction. It should also be noted that the specific potyviral gene involved in synergy is likely to be identified within the next few years. The identity of that gene is under investigation, and the search has been narrowed to three potential candidate genes on the 5'-end of the genome, the N-protease, helper component/protease, and the 50 kilodalton protein of unknown function (Vance et al., 1995). [Preliminary indications are that the single gene responsible for the synergism symptom is the helper component-protease gene in PVY and potato X potexvirus and that the *same* gene is responsible for another synergistic symptom between PVY and tobacco mosaic tobamovirus (Vance, unpublished data).]

Because synergy, unlike recombination and transcapsidation, is not related to the potential for creation of new viruses, its effects can in a sense be considered to be agronomic rather than environmental. Evaluating the potential for interactions will be an important part of assessing the agronomic performance of a transgenic crop plant, and potential interactions would likely be assessed during the standard evaluations used in cultivar development.

## C. Recombination

Recombination is defined as an exchange of nucleotide sequences between two nucleic acid molecules. Recombination between viral genomes results in heritable, permanent change. The persistence of a recombined viral genome will depend upon its fitness with respect to its ability to replicate within the original host cell, its ability to replicate in the presence of parental viruses, its ability to spread systemically within the host, or its successful transmission to other host plants.

Factors that influence recombination rates and detection of a viable recombinant include: sequence and structural similarity between the nucleic acid molecules, subcellular location and concentration of the nucleic acids, and the number of recombinational events required to form a viable recombinant viral genome (Lai, 1992). The frequency of recombination between two naturally occurring viruses or two viral

strains in field-grown plants in the absence of selection pressure has not been determined (Henry et al., 1995), and is difficult or impossible to measure meaningfully. Recombination is hypothesised as an important mechanism for virus change over evolutionary time frames, during which they may have been quite frequent (Simon and Bujarski, 1994). Recently, the nucleotide sequences of numerous viral strains from many of the known genera have been published. Sequencing data have shown that certain genes in quite different taxa probably arose from recombinational events. In other cases, a single strain of a virus has been found to contain sequences apparently derived from a virus for a different taxa, while all other closely related strains do not have these sequences. [Listing all these events is outside the scope of this document. However, several references can provide readers additional information (Koonin and Dolja, 1993; Murphy et al., 1995; Sano et al., 1992; Edwards et al., 1992; LeGall et al., 1995; Pappu et al., 1994; Goulden et al., 1991; Mayo and Jolly, 1991; Revers et al., 1995; Gibbs and Cooper, 1995]. Currently, it is not possible to determine whether these recombinational events occurred, since for example the development of modern agricultural cropping practices or in much longer time frames. However, there is evidence of virus genome stability in shorter time frames, *i.e.* since the establishment of plant virology as a science. First, the biological properties of TMV have remained remarkably stable over the past century (Ford and Tolin, 1983; Dawson, 1992); and second, the Dutch and Wisconsin (U.S.A.) substrains of alfalfa mosaic alfalmovirus strain 425 have acquired, in approximately 20 years of laboratory use in each country, several nucleotide changes leading to five amino acid changes with apparently no significant changes in biological properties (Jaspars, 1985).

The potential use of virus resistant transgenic plants in agriculture highlights the following questions regarding recombination when transgenic plants are used:

- a. Will the overall rate of viral recombination in nature be increased when these transgenic plants are used because there will be increased opportunity for recombination?
- b. What factors may affect the rate of recombination, and will that rate be proportional to the concentrations to parent molecules?
- c. Are any recombinants thus formed likely to be successful in competition with parental viruses?

Most transgenic plants are likely to be engineered in the near term with CP genes from viruses that regularly infect the host plant, because damage by those viruses poses the most constant potential for loss in the crop species. Sequences from those viruses, when available for recombination, would be unlikely to pose the potential for generating *novel* recombinants in comparison with natural mixed infections in the recipient plant, given certain conditions described below. (Genes from viruses that do not regularly infect the host plant might sometimes be introduced for experimental or other purposes, and the arguments herein would not necessarily apply in those instances.) In most virus resistant plants that have been experimentally engineered to date, transgenes that yield effective resistance to a target virus are usually expressed at very low levels compared with the levels seen in virus-infected plants. For example, in Asgrow's ZW20 squash, infected non-transgenic squash plants had a 100-fold higher concentration of viral RNA than the corresponding CP-transformed ZW20 plants. It is unlikely, though not impossible, that any compelling reason will emerge for scientists or breeders to develop new plant varieties in which high levels of transgene products are expressed, inasmuch as low level expression appears effective in conferring virus resistance. With regard to this issue, the AIBS report notes: "The implications of these low expression levels for recombination are not clear. Even assuming that the higher concentration of transgene RNA the greater the chance for recombination, we do not know what a meaningful range is; what are low and high concentrations of transgene transcript relative to unacceptable recombination rates? Currently, this information (concentration of transgene RNA) is of no use to regulatory agencies because there is no way to factor concentrations of RNA or protein into risk determination in a meaningful manner" (AIBS, 1995).

Even given these quantitative uncertainties, however, the type of background information about virus identity, environment, and disease pressure characterisation indicated in Section III above is helpful.

The use of CP gene-mediated resistance might open the possibility of novel interactions between tissue-specific viruses and other viruses. In cases where the plant is systemically infected (*i.e.* virus can be found in all cell types), the cellular location of the transgene is probably not a major issue. In contrast, if a coat protein transgene from a phloem-limited virus is used for resistance, this might increase the probability of new interactions between the transgene transcript or its gene product and other viruses that replicate only in non-phloem tissues. These new interactions may result in modified symptoms, insect transmission of the infecting virus, or modified movement of the infecting virus within the transgenic plant. However, unless a recombinational event occurred between the transgene and the infecting virus and the resulting recombinant virus was competitive, the effect would be limited and restricted to the transgenic crop. If viral infections that result in subliminal infections are known in this crop, the interactions of the transgene with these viruses in terms of important parameters (movement, symptoms, and insect transmission) can be evaluated experimentally.

**Table 2.1 Reported Viral Synergisms**

Potyviral Synergistic Interactions		References
Potato Y potyvirus (PVY)	Potato X potexvirus (PVX)	Rochow, W. F., Ross, A. F. 1955. Plant Disease (Reporter) 52:344-358.
Tobacco vein mottling potyvirus	PVX	Vance, V., B. Berger, P. H., Carrington, J. C., Hunt, A. G., Shi, X. M. 1995. Virology 206:583-590.
Tobacco etch potyvirus (TEV)	PVX	see above
Pepper mottle potyvirus	PVX	see above
Blackeye cowpea mosaic potyvirus	Cucumber mosaic cucumovirus (CMV)	Pio-Ribeiro, G., Wyatt, S. D., Kuhn, C.W. 1978. Phytopathology 68: 1260-1265.
Cowpea aphid borne potyvirus	CMV	Fisher, H. U., Lockhart, B. E. 1976. Phytopathology Z. 85:132-138.
Bean yellow mosaic potyvirus	CMV	Harrison, A. N., Gudauskas, R. T. 1968. Plant Disease (Reporter) 52:509-511.
Zucchini yellow mosaic potyvirus	CMV	Poolpol, P., Inouye, T. 1968. Annal Phytopathology Society of Japan 52:22-30.
Soybean mosaic potyvirus	Bean pod mottle comovirus	Calvert, L. A., Ghabrial, S. A. 1983. Phytopathology 73:992-997. Les, Y-S., Ross, J. P. 1968. Phytopathology 62:839-845. Quiniones, S. S., Dunleavy, J. M. 1971. Phytopathology 763-766. Ross, J. P. 1968. Plant Disease (Reporter) 52:344-348.
SMV	Cowpea mosaic comovirus	Anjos, J. R., Jarlfors, U., Ghabrial, S. A. 1992. Phytopathology 82:17-23.
Maize dwarf mosaic potyvirus	Maize chlorotic mottle virus? (MCMV)	Goldberg, K-B., Brakke, M. K. 1987. Phytopathology 77:162- 177. Niblett, C. I., Claflin, L. E. 977. Plant Disease (Reporter) 62:15-19. Uyemoto, J. K., Claflin, L. E., Wilson, D. L., Raney, R. J. 1981. Plant Disease 65:39-41.
Wheat streak mosaic potyvirus	MCMV	see above
PVY	TMV	Clark, R. L., Hill, J. H., Ellis, M. D. 1980. Phytopathology 70:131-134.
Turnip mosaic potyvirus	Cauliflower mosaic caulimovirus	Kahn, M. A., Demski, J. W. 1982. Plant Disease 66:253-256.
MDMV	Barley yellow dwarf luteovirus	Belli, G., Cinquanta, S., Soneini, C. 1980. Rivista Pathol. Veg. 16:83-86.
TEV	Dodder latent mosaic virus	Bennett, C. W. 1949. Phytopathology 39:637-646.
Non-potyviral Synergistic Interactions		
TMV	PVX	Vanterpool, T. C. 1926. Phytopathology 16:311-331.
TMV	CMV	Garces-Orejuela, C., Pound, G. S. 1957. Phytopathology 47:232-239.
TMV	Tobacco ringspot nepovirus	see above
TMV	Tomato aspermy cucumovirus	Holmes, F. O. 1956. Virology 611-617.
Cowpea chlorotic mottle bromovirus	Southern bean mosaic sobemovirus	Kuhn, C. W., Dawson, W. O. 1973. Phytopathology 63:1380-1385.
Alfalfa mosaic alfamovirus	potato acuba potexvirus	Kassanis, B. 1963. Advances in Virus Research 66:253-256.

There have been attempts to use transgenic plants to estimate experimentally the potential frequency of recombination between the transgene mRNA and the genome of a challenging virus, and/or to determine the rate of recombination between two viruses (or two viral strains). In transgenic plants expressing sequences derived from either a DNA virus (Schoelz and Wintermantel, 1993) or RNA virus (Greene and Allison, 1994), it has been demonstrated in some experiments that recombination between a viral transgene and a defective challenge virus can restore a functional, infective virus under high selection pressure. These results demonstrate that recombinational events can eventually occur in plants expressing viral sequences when inoculated with defective viruses. Because of the great interest in this area, it is expected that in the next several years additional information on the factors that influence recombination will be better understood. The results of all experiments dealing with recombination must be interpreted carefully before conclusions can be drawn, since no single experimental design is ideal to address each potential environmental condition, and each virus taxon, and certain assumptions and conditions are part of each experimental design. Some points to consider in interpreting these experiments are:

- a. Are the transgenic plants susceptible or resistant to viral infection? Some scientists have developed experimental systems to study recombination, in which the transgenic plants expressing a viral sequence are susceptible to infection by the virus which provides the transgene. In a susceptible transgenic plant the amount of viral RNA from the infecting virus would be greater than in a resistant plant; thus, higher concentrations of RNA might increase the likelihood of recombination in the experimental system. Most, if not all, transgenic plants containing CP genes that are commercialised are likely to be resistant to infection by the virus (or strain) that provided the transgene sequence.
- b. What is the selection pressure in the experiment? The AIBS report (1995) provided the following definition: "[H]igh selection pressure is defined as conditions that favour the recombinant virus, for example, a situation where the virus is not viable unless a recombination event occurs. Low selection pressure would be a situation where the novel phenotype does not confer a competitive advantage to the recombinant under the conditions of the experiment." A clear understanding of selection pressure in the experiment between a viral transgene and an infecting virus is important, since the recombination rate must be compared to natural recombination rates between the two viruses (or strains) to provide a meaningful comparison. The natural recombination rates between two viruses (or strains) may be high or low.
- c. Were the experiments performed in the natural hosts for the viruses? If a recombinant virus is formed, is it competitive with wild-type virus? Recombination rates may be affected by the host organism (Lai, 1992). The host plant also affects the mutation rates of the infecting virus (Dawson, 1992). Often, virologists have used *Nicotiana* species as experimental hosts because they are easy to transform and grow, although they are not the natural hosts of the viruses being studied. As one example, the natural host range of cauliflower mosaic virus is limited to the Brassica family (Matthews, 1991), but experiments on this virus have been performed in Solanaceous plants (Takahashi *et al.*, 1989; Baughman *et al.*, 1989; Schoelz and Shepherd, 1988). However, recombinant viruses can frequently be observed to have increased virulence (*i.e.* more severe symptoms) on model host plants that are not the natural host of either viral parent (infecting virus or virus that the transgene was derived from). If a recombinant virus is generated, determining whether it is competitive with wild type virus in the natural host of the infecting virus and the virus that provided the transgene sequence is most relevant.
- d. Do experiments performed in field situations provide additional benefits as compared to laboratory or greenhouse experiments? Whether there is any logistical or conceptual advantage for a field experiment versus a test under contained conditions depends on the experiment. However, in a field test plants are grown under natural stresses that would be found in a

commercialised crop, including inoculation of the plants by vectors containing widely prevalent viral strains of that locality, and the presence of other diseases and pests, including other viruses, etc.

If a recombinant virus is formed in a cell (either in a transgenic plant or during a mixed infection), will that recombinant participate in the replication process in that cell, move systemically in the plant, or cause a new disease? The vast majority of progeny viruses do not apparently function in the replication process. For many viruses, the RNA is encapsidated by CP, viral RNA synthesis in the cell ceases or declines to undetectable levels, and, depending on the virus and whether it is transmitted to another plant or via progeny, is degraded when the plant cell dies (Matthews, 1991). The likelihood of a recombinant becoming established depends on many factors, including its competitiveness with infecting virus and other viruses that naturally infect the plant, and all the additional factors that may affect selection pressure (e.g. temperature, vectors, host plants). Thus, to predict the probability of development of new virus disease resulting from recombination of two viruses, or between a virus and a viral derived transgene, requires a considerable level of understanding of the population biology of viruses in cells and virus movement within plants, and a better understanding of the mechanisms of how viruses cause disease.

Much of the discussion of formation of recombinant virus or the detection of new viral strains may leave the impression that a strain of virus is homogenous with respect to plant-induced symptoms or nucleotide sequence. All the single-stranded RNA genomes that have been examined have been found to exist not as a unique nucleotide sequence, but as a collection of related sequence variants around a consensus sequence. This sequence microheterogeneity is always present in natural populations (Holland *et al.*, 1982; Domingo *et al.*, 1985; Morsch *et al.*, 1988). This microheterogeneity in viral sequence has led to the concept of "quasi-species" for some viruses (Eigen, 1993). It is thought to be a result of the lack of proof-reading function in the viral replicases and of the large quantity of viral RNA produced per cell.

Most variants have one or two nucleotide changes, although some viruses (e.g. soilborne wheat mosaic furovirus) are known to have large deletions in some genes (Matthews, 1991). Variants can also be detected by changes in symptomatology. A PVX strain that produces chlorotic local lesions on tobacco plants frequently gave rise to ring spot local lesion production (Matthews, 1949). A tobacco necrosis necrovirus strain that produced white lesions on cowpea frequently gave rise to strains giving red lesions (Fulton, 1952). Thus, the microheterogeneity of viral RNA may result in sequence variation with no visible differences to major symptom alterations. Of course, even more variability in both sequence and plant-induced symptoms exists in a single virus because many viruses have well-characterised, stable strains that are sufficiently different to have been given a unique identifier (Matthews, 1991).

Although additional research is currently being funded on viral recombination, reports in two OECD Member countries have reached certain conclusions about the potential risk concerns posed by the appearance of new viruses. The conclusions reached in these two countries may not necessarily apply to all Member countries. In a report to Agricultural and Agri-Food Canada, Rochon *et al.* (1995) conclude: "It is likely that current means of detecting and controlling new diseases in this country would be adequate to control any new virus resulting from recombination between a transgene and another virus." The AIBS report to USDA (1995) concludes by stating: "With or without the use of transgenic plants, new plant virus diseases will develop that will require attention."

Undoubtedly, many new crop varieties will need to be developed to resist emerging viruses or new strains of existing viruses. The appropriate application of scientific analysis to ensure the biosafety of new varieties will allow effective control of these diseases while protecting long-term agricultural productivity and the environment.

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## SECTION 2

### GENERAL INFORMATION CONCERNING THE GENES AND THEIR ENZYMES THAT CONFER TOLERANCE TO GLYPHOSATE HERBICIDE

#### Summary Note

This document summarises the information available on the source of the genes that have been used to construct glyphosate-tolerant transgenic plants, the nature of the enzymes they encode, and the effects of the enzymes on the plant's metabolism.

**Scope of this document:** OECD Member countries agreed to limit this document to a discussion of the introduced genes and resulting enzymes that confer glyphosate tolerance to plants. The document is not intended to be an encyclopaedic review of all scientific experimentation with glyphosate-tolerant plants. In addition, this document does not discuss the wealth of information available on the herbicide glyphosate itself or the uses of the herbicide in agricultural and other applications. Food safety aspects of the use of glyphosate on glyphosate-tolerant transgenic plants are not discussed. Such information is available from other sources, including the respective governmental organisations which regulate the use of the herbicide.

While the focus of this document is on the genes and enzymes involved in encoding glyphosate tolerance, reference is not made to specific plant species into which glyphosate tolerance might be introduced. Any issues relating to the cultivation of glyphosate-tolerant plants or to the potential for, or potential effects of, gene transfer from a glyphosate-tolerant plant to another crop plant or to a wild relative are outside the agreed scope of this document. It is intended, however, that this document should be used in conjunction with specific plant species biology Consensus Documents (see list of publications at the front of the document) when a biosafety assessment is made of plants with novel glyphosate herbicide resistance.

#### 1. Herbicide Tolerance

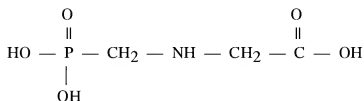
Many herbicides kill plants by interfering with enzyme function in the plant. Most of these herbicides exert their effect on a single enzyme which catalyses a key metabolic reaction in the plant. In general, plants exhibit a range of sensitivities to the herbicides used in agriculture, with some species exhibiting considerable tolerance to a single herbicide. There are several mechanisms by which plants can tolerate exposure to herbicide: (1) the plant produces an enzyme which detoxifies the herbicide; (2) the plant produces an altered target enzyme which is not affected by the herbicide; or (3) the plant produces physical or physiological barriers to uptake of the herbicide into the plant tissues and cells (Devine *et al.*, 1993).

#### 2. Glyphosate as a Herbicide

Glyphosate is widely used as a broad-spectrum weed control agent and is registered in many countries (Duke 1996, Shah *et al.* 1986). Even though glyphosate is a reversible competitive inhibitor of the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS) with respect to phosphoenolpyruvic acid (PEP), it does not inhibit any other PEP-dependent enzymatic reactions. It is a non-competitive inhibitor of EPSPS with respect to 3-phosphoshikimic acid (Steinrucken *et al.* 1984). Glyphosate is produced by

chemical synthesis. It is not a natural product. Chemically, glyphosate is N-phosphonomethyl-glycine (see Figure 2.1). Glyphosate is the active ingredient of the herbicide Roundup<sup>®</sup> (Monsanto).

Figure 2.1 Glyphosate Structure



The high sensitivity of crop plants to glyphosate has limited its use as a pre-crop emergence herbicide in no-till management strategies, and as a herbicide and crop desiccant when applied shortly before crop harvest. With the development of genetically engineered crop plants that are resistant to glyphosate, this herbicide can instead be applied after both crops and weeds have emerged, with little or no damage to the crop.

Glyphosate interferes with normal plant metabolism through inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS). In plants and micro-organisms, EPSPS is involved in the biosynthesis of aromatic amino acids, vitamins, and many secondary metabolites. It is not present in animals (Levin and Sprinson 1964, Steinrucken and Amrhein 1980). In plants, EPSPS is localised within plastids. This enzyme condenses phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid. As a consequence of the inhibition of aromatic amino acid biosynthesis, protein synthesis is disrupted, resulting in the plant's death (Kishore and Shah 1988). While some of the downstream products of the EPSPS reaction, amino acids and vitamins, are strictly essential for the growth of all living organisms, some secondary metabolites derived from the shikimate pathway may have specific survival value for the producing organism (Malik 1986). The enzyme has rigid specificity towards its substrates, which are shikimate-3-phosphate and phosphoenolpyruvate (Anderson and Johnson 1990). The reaction product, 5-enolpyruvylshikimate-3-phosphate (EPSP), is further acted upon by other enzymes to yield chorismic acid, which gives anthranilic acid (a precursor of tryptophan) and, on rearrangement, prephenic acid (a precursor of phenylalanine and tyrosine).

Based on the knowledge of the mode of action of glyphosate, several strategies have emerged for developing plants that are tolerant of exposure to the herbicide. The two successful strategies to produce glyphosate-tolerant plants are introduction of glyphosate-tolerant EPSPS and introduction of an enzyme that inactivates glyphosate, glyphosate oxidoreductase (GOX). Recombinant DNA techniques have been used to express genes that encode glyphosate-tolerant EPSPS enzyme alone or a combination of EPSPS and GOX genes in susceptible plants (Nida *et al.* 1996, Padgett *et al.* 1995, 1996).

### 3. The Development of Glyphosate-Tolerance Plants

Scientists have been unsuccessful in producing glyphosate-tolerant plants using classical techniques. Traditional mutagenesis and selection techniques have to date failed to produce a useful level of tolerance in crop plant species, although such an approach could yield a mutant form of the target enzyme that is tolerant of the herbicide but retains its desirable enzymatic function. Plant breeders also have been unable to develop glyphosate-tolerant crops using the standard techniques in which chemical or radiation exposure of seeds generates mutations in the plant genome. In cases where the desired phenotype is herbicide tolerance, spraying seedlings in the growth chamber or field can sometimes be used with success to select tolerant individual plants from millions of mutagenised individuals. Even though this approach has been used in the commercial development of imidazolinone-tolerant maize and soybean cultivars, it has not been successful in producing glyphosate-tolerant plants. This is because all mutant EPSPS, in parallel to

glyphosate tolerance, has decreased affinity for phosphoenolpyruvate. This has resulted in glyphosate-tolerant plants that have invariably shown reduced biosynthesis of aromatic amino acids.

Recombinant DNA techniques have been used to confer glyphosate tolerance to a variety of crop plant species. In this approach, plants have been transformed with genes that encode a glyphosate-tolerant enzyme that is not inhibited by glyphosate but provides substrates for the biosynthesis of amino acids. In some cases, the tolerance imparted by this gene has been further augmented by expressing a second gene that encodes the enzyme glyphosate oxidoreductase (GOX) to detoxify glyphosate (Padgett *et al.* 1996, Shah *et al.* 1986).

#### 4. Genes and Enzymes that Confer Glyphosate Tolerance

Three genes which provide field-level tolerance to glyphosate, the active ingredient in Roundup® herbicide, have been introduced into commercial cultivars. The first glyphosate-tolerant EPSPS gene was isolated from a soil bacterium, *Agrobacterium* (Barry *et al.* 1994, Duke 1996). The EPSPS synthase from this *Agrobacterium* was highly tolerant to glyphosate. When it is expressed in transgenic plants, the EPSPS encoded by this *Agrobacterium* gene fulfills the aromatic amino acid needs of the plant in the presence of glyphosate, whereas the plant version of this enzyme (ubiquitous in nature) is sensitive to glyphosate. *Agrobacterium* spp. are not human or animal pathogens, but some species are pathogenic to plants (Croon 1996, Holt 1984).

Recently, the EPSPS gene from corn (*Zea mays*) has been mutagenized *in vitro* to obtain a glyphosate-tolerant enzyme. The tolerant version of the enzyme produced by the modified maize gene is 99.3% identical to the parent enzyme (Monsanto 1997).

Also, a gene that encodes for a glyphosate-degrading enzyme called glyphosate oxidoreductase (GOX) was isolated from *Achromobacter* strain LBAA, a soil bacterium ubiquitous in nature (Barry *et al.* 1994). The encoded enzyme deactivates the herbicidal effect of glyphosate. Glyphosate oxidoreductase catalyses the conversion of glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate. GOX requires flavin adenine dinucleotide (FAD) and magnesium for activity; therefore, it is more appropriately designated an apoenzyme.

EPSPS enzyme, the target of glyphosate action, is synthesised in the cytoplasm and then transported to the chloroplast (Kishore and Shah 1988). The translocation of the protein to the chloroplast is carried out by an N-terminal protein sequence called the chloroplast transit peptide (CTP). CTPs are typically cleaved from a mature protein and degraded following delivery to the plastid (Della-Cioppa *et al.* 1986). A plant-derived coding sequence expressing a chloroplast transit peptide is often linked with each of the genes imparting glyphosate tolerance. This peptide facilitates the import of the newly translated enzymes into the chloroplasts, the site of both the shikimate pathway and glyphosate mode of action.

Use of the technology achieving transgene expression in plants is now routine. In order to achieve efficient expression of bacterial genes within plants, it has been common for researchers to modify the codon usage pattern of genes of bacterial origin prior to introducing them into plants. In this case, the codon usage pattern of the *Agrobacterium* glyphosate-tolerant EPSPS gene and glyphosate oxidase genes of *Achromobacter* have been chemically synthesised for codon optimisation for efficient expression in the plant. The amino acid sequence of the resulting enzymes is not changed. The genes associated with their transit peptide coding sequence are usually linked to other regulatory sequences like promoters, terminators, enhancers and introns. These regulatory sequences do not usually encode for a protein (Croon 1996).

These genes have been engineered (singly or in combination) into many plant species for the development of glyphosate tolerance and for use as selectable markers for identification of transformed plants. Plants field-tested with these genes include: *Beta vulgaris* (beet), *Zea mays* (corn), *Gossypium hirsutum* (cotton), *Lactuca sativa* (lettuce), *Populus* (poplar), *Solanum tuberosum* (potato), *Brassica napus* (oilseed rape, rapeseed, canola), *Glycine max* (soybean), *Nicotiana tabacum* (tobacco), *Lycopersicon esculentum* (tomato), *Triticum aestivum* (wheat).

OECD Member countries have governmental organisations which regulate the field-testing and unrestricted release of genetically engineered plants. Information about these plants is shared among various Member countries. The OECD sponsors an electronic database format for the exchange of this information. The database information is periodically updated to provide information that is both current and accurate ([www.oecd.org/ehs/service.htm](http://www.oecd.org/ehs/service.htm)).

## 5. Effect of Transgene Expression in Plants

During the life cycle of any herbicide-tolerant plant, the plant is exposed only rarely to the herbicide. Except for the production of the enzyme(s) encoding glyphosate tolerance, there should be no other changes in plant metabolism. After glyphosate application, the enzyme activities expressed by the transgenes enable the plant to survive herbicide exposure. In the case of introduced EPSPS, no new metabolic products are formed since the only difference from the native enzyme is its insensitivity to glyphosate. However, if very high expression levels result from the insertion, the levels of downstream metabolites might change. In contrast, GOX will convert glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate when glyphosate herbicide is applied (Torstensson 1985). Since glyoxylate is a naturally occurring plant metabolite involved in carbon cycling, it will be further metabolised to provide intermediates for the Krebs cycle. Since GOX is highly specific for its substrate, glyphosate, in the absence of glyphosate no metabolites are expected. The United States Environmental Protection Agency has decided that only glyphosate residues are to be regulated in plant and animal commodities, and that the major metabolite AMPA is not of toxicological concern regardless of its level in food (US EPA 1997). Information regarding decisions concerning glyphosate herbicide-tolerant plants can be found at:

<http://www.olis.oecd.org/bioprod.nsf>

[http://www.cfia-acia.agr.ca/english/plant/pbo/home\\_e.html](http://www.cfia-acia.agr.ca/english/plant/pbo/home_e.html) (Canada)

<http://ss.s.affrc.go.jp/docs/sentan/eguide/commerc.htm> (Japan)

<http://www.aphis.usda.gov/biotech/petday.html> (USA)

[http://europa.eu.int/comm/dg24/health/sc/scp/outcome\\_en.html](http://europa.eu.int/comm/dg24/health/sc/scp/outcome_en.html) (European Commission)

Western blot and enzymatic activity assays indicate that EPSPS protein from *Agrobacterium* strain CP4 is readily degraded in less than two minutes by incubation in simulated gastric fluid. In simulated intestinal fluid the enzyme activity and immunoreactivity lasts longer, being still detectable at ten minutes but undetectable at 270 minutes. The GOX protein is rapidly degraded in simulated gastric fluid and simulated intestinal fluid. After a 15 second incubation in gastric fluid, GOX has less than 90% of its initial protein epitopes as assayed by Western blot analysis, and enzyme activity loss is also greater than 90% when assayed after one minute incubation in gastric fluid. Similar results are seen in simulated intestinal fluid (US EPA 1996 and 1997).

Expression of GOX and glyphosate-tolerant EPSPS is not detrimental to plant growth, since such crops have agronomic performance similar to their parents. Governmental regulatory agencies in the United States (US Department of Agriculture 1994, 1995, 1997), Canada (Agriculture and AgriFood Canada 1995, 1996), Japan (Ministry of Agriculture, Forestry and Fisheries 1996) and European Union (European Commission 1998a, b) have made decisions that the presence of the EPSPS and GOX proteins in plants does not result in plants that are unsafe in their environments. Several lines of evidence support

the conclusion that these enzymes show low mammalian toxicity: (1) Neither enzyme shows amino acid homology to known allergens or mammalian toxins (Burke and Fuchs 1996); (2) Data from acute oral toxicity tests at high concentration of enzymes showed no toxicity (Harrison *et al.* 1996). In acute oral toxicity tests of bacterially derived CP4 EPSPS protein, no test substance adverse effects occurred at a dose of 572 milligrams per kilogram body weight (mg/kg) of the test animals. The acute toxicity of bacterially derived GOX protein showed no test substance adverse effects at doses of 91.3 mg/kg of the test animals; (3) Both enzymes are readily inactivated by heat or mild acidic conditions and are readily degraded in an *in vitro* digestibility assay which is consistent with the lack of oral toxicity (US EPA 1996, 1997). That the two enzymes show little if any toxicity is consistent with the observation that most enzymes are not considered toxic to vertebrates (Kessler *et al.* 1992). Notable exceptions are diphtheria toxin and certain enzymes in the venom of snakes, with very different exposure scenarios.

Governmental regulatory agencies in the United States (US Food and Drug Administration 1996), Canada (Agriculture and AgriFood Canada 1995, 1996), Japan and the European Union have made decisions that the presence of the EPSPS and GOX proteins in plants released into the environment do not pose a significant allergenicity risk. Two independent lines of evidence support the decision that these enzymes are not potential allergens: (1) Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid and proteases, are glycosylated, and are present at high concentrations in food. The EPSPS and GOX proteins are rapidly degraded by gastric fluid *in vitro* and are non-glycosylated. Thus, the potential for these proteins to be food allergens is minimal (Astwood *et al.* 1996, Burke and Fuchs 1996); (2) It is possible to utilise international gene databases to compare the gene sequences of a protein with other genes that encode known allergens. None of the amino acid sequences of known allergens or proteins involved in disease were shown to have similarity to the EPSPS or GOX proteins, as defined by eight identical and contiguous amino acids in a sequence. Likewise, none of the amino acid sequences of known allergens or proteins involved in coeliac disease were shown to have similarity to the GOX protein as defined by eight contiguous amino acids in a sequence (US EPA 1997).

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*SECTION 3*  
**GENERAL INFORMATION CONCERNING THE GENES AND THEIR ENZYMES THAT  
CONFER TOLERANCE TO PHOSPHINOTHRICIN HERBICIDE**

**Summary Note**

This document summarises the information available on the source of the genes that have been used to construct phosphinothricin tolerant transgenic plants, the nature of the enzymes they encode, and the effects of the enzymes on the plant's metabolism.

**Scope of this document:** OECD Member countries agreed to limit this document to a discussion of the introduced genes and resulting enzymes that confer phosphinothricin tolerance to plants. The document is not intended to be an encyclopaedic review of all scientific experimentation with phosphinothricin tolerant plants. In addition, this document does not discuss the wealth of information available on the herbicide phosphinothricin itself or the uses of the herbicide in agricultural and other applications. Food safety aspects of the use of phosphinothricin on phosphinothricin tolerant transgenic plants are not discussed. Such information is available from other sources, including the respective governmental organisations which regulate the use of the herbicide.

While the focus of this document is on the genes and enzymes involved in encoding phosphinothricin tolerance, reference is not made to specific plant species into which phosphinothricin tolerance might be introduced. Any issues relating to the cultivation of phosphinothricin tolerant plants or to the potential for, or potential effects of, gene transfer from a phosphinothricin tolerant plant to another crop plant or to a wild relative are outside the agreed scope of this document. It is intended, however, that this document should be used in conjunction with specific plant species biology Consensus Documents (see list of publications at the front of the document) when a biosafety assessment is made of plants with novel phosphinothricin herbicide resistance.

## **1. Herbicide Tolerance**

Many herbicides kill plants by interfering with enzyme function in the plant. Enzymes are the proteins which catalyse the diverse reactions which comprise the plant's metabolism. Some herbicides exert their effect on a single enzyme which catalyses a key metabolic reaction in the plant. In general, plants exhibit a range of sensitivities to the herbicides used in agriculture, with some species exhibiting considerable tolerance to a herbicide. There are several mechanisms by which plants can tolerate exposure to herbicide: (1) the plant produces an enzyme which detoxifies the herbicide, (2) the plant produces an altered target enzyme which is not affected by the herbicide, or (3) the plant produces physical or physiological barriers to uptake of the herbicide into the plant tissues and cells (Devine *et al.* 1993).

Phosphinothricin tolerance has been conferred to a variety of plant species (see Section V) by using recombinant DNA techniques to transfer one of two genes (*pat* or *bar*) from bacteria to enable the plant to produce an enzyme (phosphinothricin acetyl transferase; PAT). Expression of PAT within the plant cell detoxifies L-PPT, a herbicide (the L-isomer of phosphinothricin), and thereby makes the plant tolerant to L-PPT. This document summarises the information available on the source of these genes, the nature of the enzymes they encode, and the consequences of transgene expression in the plant. Finally, it is suggested

that the reader visit the OECD BioTrack Online website to see the current status of phosphinothricin tolerant plants that have been released under small-scale experimental field trial conditions (<http://www.olis.oecd.org/biotrack.nsf>) and those that have been approved for commercial release (<http://www.olis.oecd.org/bioprod.nsf>).

## 2. Phosphinothricin as a Herbicide

### A. The herbicide phosphinothricin

Phosphinothricin is the amino acid, 4-[hydroxy-(methyl) phosphinoyl]-D,L-homoalanine. The L-isomer of phosphinothricin (L-PPT) is widely used as a broad-spectrum weed control agent and is registered for use as a herbicide in many countries. The D-isomer, D-PPT, exhibits no herbicidal activity. L-PPT is the active ingredient of the herbicide glufosinate ammonium. Glufosinate ammonium is an equimolar, racemic mixture of the D- and L-isomers of PPT. Although D-PPT is not herbicidal, L-PPT inhibits glutamine synthetase of susceptible plants and results in the accumulation of lethal levels of ammonia. L-PPT is considered a broad-spectrum herbicide because it is herbicidal to a wide range of plant species. Some plant species exhibit greater sensitivities than others. Additional information on the properties and use of the herbicide phosphinothricin can be obtained from the governmental authorities which regulate its use. For example, the United States Environmental Protection Agency regulates herbicide use and maintains health assessment information concerning phosphinothricin (glufosinate ammonium) available on the Internet (<http://www.epa.gov/ngispgm3/subst/irisbak/0247.htm>).

### B. Production of L-PPT by micro-organisms

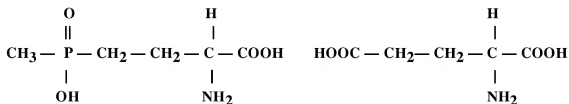
Species of the genera *Streptomyces* and *Kitasatosporia* are the only organisms reported to synthesise the amino acid L-PPT. Species of these genera are Gram-positive, sporulating soil micro-organisms, commonly referred to as actinomycetes (Cross 1989, Locci 1989).

L-PPT has been reported as a component of only two tripeptides, bialaphos and phosalone (Wild and Ziegler 1989, Omura *et al.* 1984). Bialaphos is a tripeptide (phosphinothricyl-L-alanyl-L-alanine) produced naturally by *Streptomyces hygroscopicus* and *S. viridochromogenes*. Each molecule of bialaphos comprises L-PPT and two residues of alanine. Phosalone is a tripeptide (phosphinothricyl-L-alanyl-L-leucine) produced by *Kitasatosporia phosalonea* (Takahashi *et al.* 1984). Peptidase activity readily breaks the peptide bonds, liberating the L-PPT moiety from either bialaphos or phosalone (Thompson *et al.* 1987, Wild and Ziegler 1989, Omura *et al.* 1984).

L-PPT is the active ingredient in a number of commercial herbicide formulations. The L-PPT can be derived either from fermentation cultures that yield bialaphos, or from chemical synthesis of glufosinate ammonium. Glufosinate ammonium is an equimolar racemic mixture of L-PPT and D-PPT. There are presently no commercial herbicides which use phosalone.

### C. Mode of action of L-PPT herbicides

Herbicides based on L-PPT are active against a broad spectrum of plant species. L-PPT is a structural analogue of glutamate, the substrate of glutamine synthetase (see the side-by-side comparison of L-PPT and glutamate in Figure 2.2). L-PPT exerts its herbicidal effect through the inhibition of glutamine synthetase (Bayer *et al.* 1972). In the presence of ATP, L-PPT inhibits glutamine synthetase irreversibly (Devine *et al.* 1993). When L-PPT inhibits glutamine synthetase, phytotoxic levels of ammonia accumulate in the plant (Mifflin and Lea 1976, Tachibana *et al.* 1986).

**Figure 2.2 L-isomer of phosphinothricin (left) compared to glutamate (right)**

Glutamine synthetase is the enzyme responsible for the synthesis of the amino acid glutamine from glutamic acid and ammonia in both eukaryotes and prokaryotes. This is the first reaction in the pathway that assimilates inorganic nitrogen into organic compounds. In plants, glutamine synthetase exists in multiple isozymic forms that can be localised within the cell in the cytosol and plastids. In addition, various isozymic forms are predominately found in certain plant tissues or organs (McNally *et al.* 1983). In plant roots, the primary role of glutamine synthetase is to assimilate ammonia. However, the glutamine synthetase in leaves is primarily responsible for the reassimilation and detoxification of ammonia (Shah *et al.* 1986, Kishore and Shah 1988). Glutamine synthetase is the only enzyme in plants that can detoxify the ammonia released by photorespiration, nitrate reduction and amino acid degradation.

As scientists have increased their understanding of the mode of action of L-PPT, several strategies have emerged for developing plants that are tolerant of exposure to the herbicide. The two most prominent strategies are (1) to identify a variant of glutamine synthetase that is insensitive to inhibition by L-PPT, and (2) to introduce a gene that encodes an enzyme designed to inactivate the herbicidal activity of L-PPT. Despite attempts to utilise the first strategy (AgrEvo 1994), to date only the second strategy has been successful in conferring tolerance to L-PPT.

### 3. The Development of L-PPT Tolerant Plants

**“Traditional” plant breeding techniques.** To date, plant breeders have not been successful in using so-called “traditional” plant techniques to develop L-PPT tolerant crop plants. Historically, plant breeders have tried to identify desirable attributes in the germplasm collection of the crop itself or among closely related plant species. The desirable trait(s) would then be bred into the crop via sexual hybridizations, some of which might require some human intervention to achieve success.

Alternatively, in the absence of finding the desired trait in germplasm collections, breeders have used chemical or radiation induced mutagenesis to create variants that would then be evaluated for efficacy and agronomic performance. This technique relies on slightly modifying the plant enzyme which is the “target” of the herbicide (*i.e.* the enzyme(s) which the herbicide inhibits). Thus, the mutagenesis results in a target enzyme that still functions but has lost its sensitivity to a herbicide. This approach has been successful in developing maize and soybean varieties which produce a form of acetolactate synthase that is no longer sensitive to imidazolinone and sulfonyleurea herbicides (Saari and Mauvais 1996, Shaner *et al.* 1996). Readers interested in an overview of techniques for producing herbicide tolerant plants may consult Dyer (1996).

Attempts to use such mutagenesis and selection techniques have also failed to produce a useful level of L-PPT tolerance in crop plant species. Included in these efforts has been a decade of failed attempts to obtain maize plants which have a glutamine synthetase that is not inhibited by L-PPT (AgrEvo 1994).

**Recombinant DNA techniques.** Over the past decade, recombinant DNA techniques have been successfully employed to confer L-PPT tolerance to a variety of crop plant species (see below). Using this approach, plants have been transformed with one of two bacterial genes (*pat* or *bar*) which encode an enzyme, phosphinothricin acetyl transferase (PAT), that detoxifies L-PPT. The expression of the PAT

enzyme in the transgenic plants has been used in three different ways: (1) to confer agronomically useful levels of L-PPT tolerance for crop production, (2) to provide a selectable genetic trait (marker) that can be used in the laboratory or field, or (3) to provide a selectable genetic trait in conjunction with a genetic male sterility system.

- *L-PPT tolerance for agronomic use.* In some plants modified to express PAT, the tolerance to L-PPT will be used agronomically in the cultivation of the crop by the grower. An example of such a transgenic L-PPT tolerant plant is the oilseed rape/canola (*Brassica napus* L.) line HCN92, which was the first L-PPT tolerant plant cleared by governmental authorities. Line HCN92 was authorised by Canadian agencies for unconfined release, food and livestock feed use in Canada in 1995 (Agriculture and Agri-Food Canada 1995a, 1995b, 1996a, 1996b). Since then, other transgenic L-PPT tolerant crop plant lines have been cleared through relevant governmental regulatory authorities. The OECD “Biotrack On-line” database (<http://www.olis.oecd.org/bioprod.nsf>) maintains an updated listing of such approvals.
- *L-PPT tolerance as a selectable marker.* In some of the plants engineered with the *pat* or *bar* gene, the gene serves as a selectable marker gene. Such plants may not necessarily express agronomically useful levels of tolerance to L-PPT. Marker genes are routinely used in developing transgenic plants because they enable the researchers to select successful transformants in the laboratory. In addition, tolerance to L-PPT can be used as a selectable marker in the field. Vasil (1996) states that, in some plant species, expression of L-PPT tolerance has been a more useful selectable marker than the kanamycin resistance that has been used since the inception of recombinant DNA research with plants. Final clearances were granted in the United States in 1995 (USDA 1995) for the first transgenic plant which utilised L-PPT tolerance (conferred by the *bar* gene) as a selectable marker trait.
- *L-PPT tolerance for selection as part of a male-sterility system.* Transformation with L-PPT can be used alone or in conjunction with other genes. An example of this is when PAT expression is also part of a genetically engineered male sterility system that can be used in the production of F<sub>1</sub> hybrid plant varieties (Mariani *et al.* 1990). In this system, plants are transformed with a genetic construct that couples genes that block pollen production, together with the selectable marker gene which confers expression of PAT. Therefore, the PAT expression in the transformed plants makes it possible to use L-PPT as part of a practical system for plant breeders to produce hybrid seed. In 1996, a maize line engineered with this male sterility system was cleared in the United States prior to commercial release (U.S. Department of Agriculture information found at <http://www.aphis.usda.gov/biotech>). Such transgenic male sterility systems are currently being employed for variety development and seed production in canola, chicory and maize.

A variety of plant species have been engineered with either the *pat* or *bar* genes, and many of these plants have been grown in small-scale field tests to evaluate performance under field conditions. As of 1997, these include: *Agrostis palustris* (creeping bentgrass), *Avena sativa* (barley), *Arachis hypogaea* (peanut), *Beta vulgaris* (sugarbeet), *Brassica oleracea* (wild cabbage), *Chichorium intybus* (chicory), *Daucus carota* (carrot), *Festuca arundinacea* (tall fescue), *Gossypium hirsutum* (cotton), *Hordeum vulgare* (barley), *Lycopersicon esculentum* (tomato), *Medicago sativa* (alfalfa), *Gladiolus* sp. (*gladiolus*), *Cucumis melo* (melon), *Populus* spp. (poplar), *Solanum tuberosum* (potato), *Brassica napus* (rapeseed), *Oryza sativa* (rice), *Glycine max* (soybean), *Sorghum bicolor* (sorghum), *Saccharum officinarum* (sugarcane), *Nicotiana tabacum* (tobacco), *Triticum aestivum* (wheat) and *Zea mays* (maize).

A number of countries have governmental organisations which regulate the field testing and unrestricted release of genetically engineered plants. Information about these plants in OECD Member

countries is available to anyone interested. The database, available on the Internet, is periodically updated to provide information that is both current and accurate (<http://www.oecd.org/ehs/service.htm>).

#### 4. Genes and Enzymes that Confer L-PPT Tolerance

##### A. Donor organisms for the genes

Two species of actinomycetes, *Streptomyces viridochromogenes* and *S. hygroscopicus*, have been the source of the genes which have been transferred to plants to confer tolerance to L-PPT (Thompson *et al.* 1987, Kumada *et al.* 1988, Hara *et al.* 1991). These species of *Streptomyces* are saprophytic, soil-borne microbes and are not considered pathogens of plants, humans, or other animals (Locci 1989, Cross 1989).

Genes encoding PAT enzymes (PATs) have been isolated from *S. viridochromogenes* and *S. hygroscopicus*. In *S. hygroscopicus*, a PAT is encoded by the *bar* (bialaphos-resistance) gene, whereas in *S. viridochromogenes* a PAT is encoded by the *pat* gene (some researchers refer to the PAT encoded by *bar* as BAR). The *pat* and *bar* genes are very similar, sharing 87 per cent homology at the nucleotide sequence level (Wohlleben *et al.* 1988, 1992). The respective PAT enzymes encoded by *pat* and *bar* are also very similar, and share 85 per cent homology at the amino acid level (Wohlleben *et al.* 1988, 1992). Wehrmann and co-workers (1996) recently published results of extensive characterisation of the PATs encoded by *bar* and *pat*. They conclude that the PATs encoded by *pat* and *bar* are so similar as to be functionally equivalent for the purpose of conferring tolerance to L-PPT.

##### B. Modification of the native gene to enable expression in plants

In order to achieve efficient expression of the *pat* and *bar* genes within plants, it has been common for researchers to modify the codon usage pattern of genes of bacterial origin prior to introducing them into plants. The *bar* and *pat* genes isolated from *Streptomyces* spp. have relatively high G:C content when compared to plant genes, and as a consequence the native microbial genes are inefficiently expressed in plants. In this case, the codon usage pattern of the native *Streptomyces* genes have been modified prior to introduction into the plant. This resulted in increased expression levels. The amino acid sequence of the resultant PAT is not changed (Eckes *et al.* 1989, USDA 1995).

Genes of bacterial origin require modification with appropriate plant-expressible regulatory sequences such as promoters, enhancers, intron and terminators. These regulatory sequences do not encode amino acids and therefore do not affect the coding region of the PAT enzyme. Further discussion on the use of regulatory sequences to achieve expression of transgenes in plants is beyond the scope of this document.

##### C. Specificity of PAT enzymatic activity

Both PAT enzymes encoded by *bar* and *pat* appear to be: (1) functionally equivalent for the purpose of conferring tolerance to L-PPT, and (2) highly specific for their substrate (Wehrmann *et al.* 1996). In the presence of acetyl-CoA as a co-substrate, PAT catalyses the acetylation of the free amino group of L-PPT to yield N-acetyl-L-PPT, a compound that does not inactivate glutamine synthetase. Both of the PAT enzymes are highly specific for L-PPT and do not acetylate other L-amino acids, nor do they acetylate D-PPT (Wehrmann *et al.* 1996, AgrEvo 1994). In the presence of excess concentrations of L-amino acids, both PATs also are unaffected in their ability to acetylate L-PPT (Wehrmann *et al.* 1996).

In L-PPT tolerant plants which express relatively high levels of PAT, the main residue metabolite of L-PPT catabolism is N-acetyl-phosphinothricin (Droege-Laser *et al.* 1994). When PAT expression is low, the degradation pathways of L-PPT can result in the residue metabolites found in L-PPT sensitive plants, namely 4-methyl-phosphinico-2-hydroxy-butanoic acid and 3-methylphosphinico-propionic acid (Droege-Laser *et al.* 1994).

## 5. Effects of Transgene Expression in Plants

During the life cycle of any herbicide tolerant plant, the plant is only rarely exposed to the herbicide. When the active herbicide L-PPT is applied to the herbicide tolerant plants, the PAT activity will enable the plant to render L-PPT non-toxic to the plant. The PAT enzyme detoxifies phosphinothricin (L-PPT) by acetylation into an inactive compound. Metabolism studies on genetically modified oilseed rape (*Brassica napus* L.) showed a rapid conversion of L-PPT to the non-toxic metabolite, N-acetyl-glufosinate (European Commission 1998). It has also been reported that PAT has extremely high substrate specificity for L-PPT and demethylphosphinothricin (DMPT) (Thompson *et al.* 1987), but experimental data have shown it cannot acetylate L-PPT's analogues L-glutamic acid, D-PPT, nor any protein or amino acid (Wehrmann *et al.* 1996, Agriculture and Agri-Food Canada 1995a, 1995b).

Expression of PAT is not detrimental to plant growth, since such crops have agronomic performance similar to their parents when engineered with either *pat* or *bar* genes. These conclusions have been described in decision documents published by regulatory authorities in Canada, the European Union and the United States prior to the commercialisation of L-PPT tolerant *Chichorium intybus* (chicory), *Brassica napus* (rapeseed, oilseed rape, canola) and *Zea mays* (maize). Information on decisions concerning phosphinothricin herbicide tolerant plants can be found at:

<http://www.olis.oecd.org/bioprod.nsf>

[http://www.cfia-acia.agr.ca/english/plant/pbo/home\\_e.html](http://www.cfia-acia.agr.ca/english/plant/pbo/home_e.html) (Canada)

<http://ss.s.affrc.go.jp/docs/sentan/eguide/commerc.htm> (Japan)

<http://www.aphis.usda.gov/biotech/petday.html> (United States)

[http://europa.eu.int/comm/dg24/health/sc/scp/outcome\\_en.html](http://europa.eu.int/comm/dg24/health/sc/scp/outcome_en.html) (European Commission)

In recent years, a number of allergenic constituents of plants have been characterised. Allergens usually share a number of characteristics, including the following: (1) they are proteins, (2) they range between 10-70 kiloDaltons in molecular weight, (3) they typically, but not absolutely, are glycosylated, (4) they are stable to digestion (peptic and tryptic conditions of the mammalian digestive system), (5) they are stable to processing, and (6) they are present as the major protein component in the specific food (Metcalf *et al.* 1996, FAO/WHO 1996, Fuchs and Atwood 1996). The PAT protein is not a known allergen. SDS-PAGE shows a molecular mass of 22-23 kD for *pat* and *bar* gene products, slightly higher than the calculated mass of 20.6 kD. Gel filtration chromatography shows activity at the 43 kD peak (homodimer) (Wehrmann *et al.* 1996). The same authors reported that when PAT and BAR proteins, produced from the *pat* and *bar* genes respectively, were subjected to simulated gastric conditions with pepsin, both proteins were degraded within seconds, and the enzymatic activity dropped to zero within a 5-15 second timeframe.

Other reported studies have shown that the enzyme was inactivated within one minute when subjected to typical mammalian stomach conditions and was inactivated during processing of canola seed (from transgenic *Brassica napus* expressing the PAT enzyme) into feed ingredients (European Commission 1998). The USEPA (1997) reported that experimental data indicated that the PAT protein is rapidly degraded in the gastric environment and is also readily denatured by heat or low pH. Many food allergens have been biochemically characterised, and databases make it possible to compare the amino acid sequence of a protein to those proteins in the database which are known to elicit allergenic responses. The nucleotide sequence of the gene was provided. When subjected to comparative analyses using the GENEBANK DNA database (Agriculture and Agri-Food Canada 1995a) and the FASTDB algorithm of Intelligenetics with three databases of polypeptide sequences (Agriculture and Agri-Food Canada 1995b), the PAT enzyme amino acid sequence did not show significant homology with other proteins present in the databases, except with other phosphinothricin acetyltransferases originating from different organisms. No resemblance with potential toxins or allergens was observed. USEPA (1997a) concluded that "the potential for the PAT protein to be a food allergen is minimal."

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjobald *et al*, 1992). There is no evidence available indicating that the PAT protein is toxic to either humans or other animals. In a 14-day feeding study using bacterially produced purified PAT enzyme, mice gavaged with high levels of the protein (5,050 milligram/kilogram bodyweight) showed no treatment-related significant toxic effects (USEPA 1995). It has also been reported that an avian dietary test was performed with the seed-eating canary bird (*Serinus canaria domestica*), and that a feeding study was performed with the domesticated rabbit (*Oryctolagus cuniculus*); these studies showed no differences in food consumption, behaviour and body weight between birds or rabbits fed with the transgenic PAT producing *Brassica napus* L. (rapeseed, oilseed rape, canola) or non-transgenic counterparts (Agriculture and Agri-Food Canada 1995b).

With respect to the toxicity of PAT, USEPA concludes that “the acute oral toxicity data submitted support the prediction that the PAT protein would be non-toxic to humans.” In the United States the EPA, based on submitted toxicological data, established an exemption from the requirement of a tolerance for residues of the plant-pesticide ingredients phosphinothricin acetyltransferase (PAT) and the genetic material necessary for its production in all plants (USEPA 1997b).

Governmental regulatory authorities in the United States, Canada, Japan and European Union have made decisions that the presence of the PAT protein in plants does not render them unsafe for consumption as food or feed (see above). Further information on the food safety criteria can be found in published regulations, guidelines and policy statements of various governmental agencies.

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SECTION 4  
**HERBICIDE BIOCHEMISTRY, HERBICIDE METABOLISM AND THE RESIDUES IN  
GLUFOSINATE-AMMONIUM (PHOSPHINOTHRICIN) – TOLERANT TRANSGENIC  
PLANTS**

**Summary Note**

This document summarises the information available on the herbicide biochemistry, the herbicide metabolism and the residues in glufosinate-ammonium (phosphinothricin)-tolerant transgenic plants.

**Scope of this document:** This document is limited to a condensed discussion of the herbicide biochemistry and metabolism specifically in glufosinate-ammonium (phosphinothricin)-tolerant transgenic plants. It is not intended to be an encyclopaedic review of all scientific experimentation with glufosinate tolerant plants or with the herbicide glufosinate itself. Especially, this document is not to be confused with the type of dossier currently composed for plant pesticides according to directive 91/414/EEC. Moreover, it does not discuss the plentiful information available on the use of the herbicide in agricultural and other applications. Food safety aspects of the use of glufosinate-ammonium on glufosinate-ammonium-tolerant plants are beyond the scope of this document. Such information is available from other sources, including the respective governmental organisations regulating herbicide use.

**1. Biochemistry and Physiology of the Herbicide in Non-tolerant and in genetically Modified Glufosinate (Phosphinothricin) – Tolerant Plants**

Glufosinate (phosphinothricin; DL-homoalanin-4-yl(methyl)phosphinic acid) is a racemic phosphinico amino acid (Hoerlein, 1994). Its ammonium salt (glufosinate-ammonium) is widely used as a non-selective herbicide and is the active ingredient of the commercial herbicide formulations Basta®, Buster®, Challenge®, Conquest®, Dash®, Final®, Finale®, Liberty® and Ignite®. The L-isomer of glufosinate is a structural analogue of glutamate and, therefore, is a competitive inhibitor of the enzyme glutamine synthetase (GS) of bacteria and plants (Bayer *et al.*, 1972; Leason *et al.*, 1982). The D-isomer is not a GS inhibitor and is not herbicidally active.

Due to the inhibition of GS, non-tolerant plant cells accumulate large amounts of toxic ammonia produced by nitrate assimilation and photorespiration (Tachibana *et al.*, 1986) and the level of available glutamine drops (Sauer *et al.*, 1987). Damage of cell membranes and inhibition of photosynthesis are followed by plant cell death. The action of glufosinate is dependent on environmental conditions. Temperatures below 10°C, as well as drought stress, reduce its efficacy because of the limited metabolic activity of the plant (Donn, 1982). Also, light is an important factor for the action of glufosinate (Koecher, 1983).

In genetically modified glufosinate-tolerant plants, the L-isomer of glufosinate is rapidly metabolized by the action of the enzyme phosphinothricin acetyltransferase (PAT) into the non-phytotoxic stable metabolite N-acetyl-L-glufosinate (2-acetamido-4-methylphosphinico-butanoic

acid). N-acetyl-L-glufosinate does not inhibit glutamine synthetase. Therefore, no phytotoxic physiological effects are observed in genetically modified glufosinate-tolerant plants.

Glufosinate is a contact herbicide and is taken up by the plant primarily through the leaves (Haas and Müller, 1986). There is no uptake from the soil through the roots, presumably because of the rapid degradation of glufosinate by soil micro-organisms. There is limited translocation of glufosinate within the plant. After application of L-glufosinate, N-acetyl-L-glufosinate and further metabolites on distinct leaves, a preferential transport into the upper leaves and a low level of translocation into the lower plant parts was observed in both genetically modified and unmodified tobacco plants (Droege, 1991; Droege-Laser *et al.*, 1994).

Glufosinate has a wide spectrum of activity encompassing monocotyledonous and dicotyledonous species. Due to its limited systemic action, there is no enduring effect on perennial weeds. Examples of weed species that are not, or only weakly, combated by glufosinate are *Viola arvensis*, *Bromus spp.*, *Lolium spp.*, *Agropyron repens* and *Urtica urens* (Hoechst, 1991). Weeds emerging after herbicide application are not affected.

Glufosinate is rapidly broken down in soil due to microbial degradation. At 20°C, the soil half-life is less than 10 days (Smith, 1988; Dorn *et al.*, 1992). Metabolites arise from oxidative deamination and from acetylation (Dorn *et al.*, 1992). L-glufosinate can be used by micro-organisms as a source of nitrogen (Tebbe and Reber, 1989). There are no special reports on the degradation of the D-enantiomer in soil, however, the fast dissipation of the DL-racemic mixture was found in all soils investigated under laboratory, as well as, field conditions (Dorn *et al.*, 1992; Smith, 1989). The end products of microbial degradation are CO<sub>2</sub> and natural phosphorus compounds. There is also formation of bound residues which are finally mineralized (Dorn *et al.*, 1992).

## 2. Metabolism of Glufosinate-ammonium in Genetically Modified Plants in Comparison to Non-Transgenic Plants

Because of the widespread use of glufosinate in agricultural practices (non-selective application, as a desiccant, selective application in tolerant crops), the metabolism of glufosinate in sensitive, as well as in glufosinate-tolerant plants, is addressed. If the PAT enzyme is used as part of selectable marker systems of genetically modified plants, lower levels of PAT activity are required compared to glufosinate-tolerant crops for selective field applications of the herbicide.

The metabolism of glufosinate in artificial systems like cell suspension cultures (soybean, wheat, maize) and sterile plants (tobacco, alfalfa, carrot) has been analyzed by Komossa and Sandermann (1992) and by Droege-Laser *et al.* (1994). After treatment of non-transgenic plants with glufosinate, the unstable intermediate 4-methylphosphinico-2-oxo-butanoic acid (PPO) is formed via deamination. A rapid decarboxylation reaction then results in the stable main metabolite 3-methylphosphinopropionic acid (MPP) which is non-phytotoxic. Within non-transgenic plants, PPO can also be reduced to form 4-methyl-phosphinico-2-hydroxy-butanoic acid, another final and stable product (Droege-Laser *et al.*, 1994). In contrast to transgenic PAT-expressing plants, there is no direct proof that in non-tolerant plants only the L-isomer is metabolized.

The metabolism of glufosinate in non-tolerant plants is only limited because plants rapidly die after herbicide application. Moreover, if used as a non-selective herbicide in agricultural practice, glufosinate is not intended to be applied directly, except for desiccation purposes. If crop plants have not emerged at the time of application, residues in the crop plants can only be due to uptake from the soil. Studies evaluating the amount and nature of "indirect" uptake have shown that traces, mainly of the major metabolite 3-methylphosphinopropionic acid (MPP), can be found (Hoerlein, 1994). This

non-phytotoxic metabolite is also a well known soil metabolite (Tebbe and Reber, 1988) which can be taken up by the roots. It was found to be the only relevant residue following normal weed control in non-transgenic plants (Hoerlein, 1994). In desiccation, residues consist of unchanged glufosinate, with small portions of MPP and a non-relevant portion of 2-methyl-phosphinico-acetic acid.

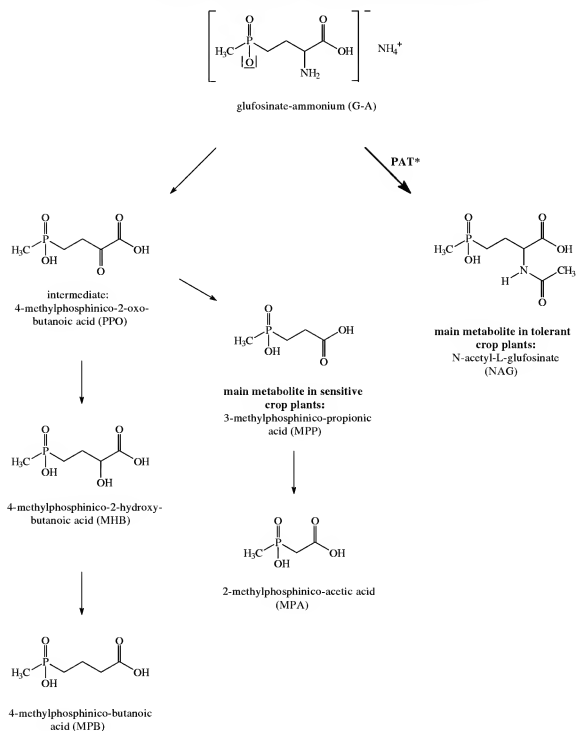
The insertion of genes encoding phosphinothricin acetyltransferase (PAT) enables plants genetically modified in this way to rapidly metabolize the herbicidal active moiety of glufosinate-ammonium into the non-phytotoxic metabolite N-acetyl-L-glufosinate (2-acetamido-4-methylphosphinico-butanoic acid). This metabolite is not found in non-transgenic plants.

The metabolism of glufosinate-ammonium following direct application on genetically modified glufosinate-tolerant corn (maize), oilseed rape (canola), tomato, soybean and sugar beet (*Figure 2.3*) has been investigated with the formulated test substance (Burnett, 1994; Tshabalala, 1993; Thalacker, 1994; Stumpf, 1995; Rupprecht and Smith, 1994; Rupprecht *et al.*, 1995; Allan, 1996). In all glufosinate-tolerant crops, the principal residues were N-acetyl-L-glufosinate and - usually with lower concentrations – glufosinate-ammonium and MPP. In corn grain and rape seed, the main residue identified was MPP, with lower concentrations of N-acetyl-L-glufosinate. In corn forage, in soybean seed, in sugar beet roots and in tomato fruit, the main residue was N-acetyl-L-glufosinate. Experiments of Droege *et al.* (1992) and Droege-Laser *et al.* (1994) using transgenic tobacco, carrot, and alfalfa plants also found N-acetyl-L-glufosinate as the major metabolite in glufosinate-tolerant plants. Besides the principal residues, trace levels of other metabolites were also identified in soybean including 2-methylphosphinico-acetic acid (MPA) and 4-methylphosphinico-butanoic acid (MPB). The herbicidally inactive D-glufosinate appears to be stable in plants due to the L-specific acetylation activity of the PAT enzyme (Droege *et al.*, 1992).

In genetically modified glufosinate-tolerant plants expressing the PAT enzyme, it appears that two metabolic routes compete: (1) the deamination of glufosinate and subsequent conversion of 4-methyl-phosphinico-2-oxo-butanoic acid (PPO) to 3-methylphosphinico-propionic acid (MPP) or to 4-methyl-phosphinico-2-hydroxy-butanoic acid, and (2) the N-acetylation of L-glufosinate by PAT (Droege-Laser *et al.*, 1994). The second of these two routes predominates when PAT specific activity is relatively high.

If genetically modified plants express the PAT enzyme at a low level, the deamination pathway with the formation of MPP predominates. In this case, besides substantial amounts of the acetylated and non-acetylated forms of L-glufosinate, the metabolites 4-methyl-phosphinico-2-oxo-butanoic acid (PPO), 3-methylphosphinico-propionic acid (MPP) and 4-methyl-phosphinico-2-hydroxy-butanoic acid are formed (Droege-Laser *et al.*, 1994).

**Figure 2.3 Metabolism of Glufosinate-Ammonium in Non-Transgenic and in Transgenic, Tolerant Crop Plants (Corn, Oilseed rape, Tomato, Soybean, Sugar beet)**



\*) PAT = phosphinothricin acetyl - transferase

Source : derived from FAO, 1998

### 3. Metabolites and Residues in Genetically modified Plants

The FAO's Joint Meeting of Experts on Pesticide Residues (JMPR) suggested, in 1998, a revised residue definition, considering the nature of the residue occurring in conventional and transgenic glufosinate-tolerant plants. This definition was confirmed by the 1999 JMPR as suitable for the establishment of maximum residue levels and for the estimation of dietary intake. For glufosinate-ammonium, residue is defined as the sum of glufosinate-ammonium, MPP and N-acetyl-L-glufosinate (FAO, 1998).

For residue studies, glufosinate-ammonium and the principal metabolites N-acetyl-glufosinate and 3-methylphosphinico-propionic acid (MPP) are extracted from finely ground sample material with water. After cleaning-up of the extracts, the residues are derivatised, resulting in the formation of methylated/acetylated derivatives. These are cleaned up and determined by gas chromatography using a phosphorus-specific flame photometric detector, yielding analytical recoveries which are satisfactory on many substrates. Glufosinate-ammonium and N-acetyl-L-glufosinate are determined as a common derivative and MPP is quantified as a separate derivative. If a differentiation between glufosinate-ammonium and N-acetyl-L-glufosinate is required, the two compounds need to be separated prior to derivatisation.

Using this procedure, the following individual total residues represented as the sum of glufosinate-ammonium, N-acetyl-L-glufosinate and MPP were obtained from genetically modified, glufosinate-tolerant plants while the limit of quantification for each analyte was 0.05 mg/kg. Individual residue data are mainly part of national submissions for glufosinate-ammonium.

#### A. Oilseed rape

At an application rate of 750 g/ha or 2 x 800 g/ha, the total residue in the seed at harvest encompasses between < 0.05 and 0.24 mg/kg. Rapeseed oil was found to contain below 0.05 mg/kg total residue.

#### B. Corn

At an application rate of 400 + 500 g/ha or 2 x 800 g/ha, the total residue in corn grain was between < 0.05 and 0.07 mg/kg. Corn oil contained less than 0.05 mg/kg total residue.

#### C. Soybean

At an application rate of 400 + 500 g/ha, the total residue in soybean seed ranged from 0.32 to 1.88 mg/kg.

#### D. Sugar beet

At an application rate of 2 x 600 g/ha or 2 x 800 g/ha, the total residue in roots which are relevant to human nutrition as a raw material for sugar production, were found to be between < 0.05 and 0.88 mg/kg. Refined sugar after processing contained no residues (< 0.05 mg/kg).

The lowest NOEL (no observed effect level), established in a chronic (24 months) feeding study in rats, was 2 mg glufosinate-ammonium/kg body weight/day (Ebert *et al.*, 1990). This low toxicity is due to the mode of action of glufosinate. In mammals, glufosinate-ammonium competitively inhibits glutamine synthetase (GS). However, contrary to the situation in plants, fixation of ammonia is guaranteed by several metabolic pathways in order to maintain homeostasis of the amino acid pool.

The biosynthesis of glutamine from glutamate forms only one of the possibilities for fixation of ammonia and amino groups. Thus GS is only of minor importance for ammonia fixation in mammals. In this context, Hack *et al.* (1994) found that inhibition of glutamine synthetase by glufosinate did not essentially affect the level of ammonia, glutamate and other amino acids. Since the toxicological data indicated no genotoxic, carcinogenic or teratogenic potential, an acceptable daily intake (ADI) value of 0.02 mg/kg body weight/day was accepted for glufosinate (WHO, 1992). This value has been confirmed as group ADI for glufosinate-ammonium, MPP and N-acetyl-L-glufosinate (WHO, 1999).

Tolerances for combined residues of glufosinate-ammonium and its metabolites (3-methylphosphinicopropionic acid and N-acetyl-L-glufosinate) have been established in the USA for transgenic field corn and transgenic soybean. The tolerances are 0.2 mg/kg and 2.0 mg/kg for corn grain and for soybean seed, respectively (EPA, 1999).

Glufosinate-ammonium is registered for the use in the following transgenic tolerant crops:

Canada	Canola and Corn
USA	Corn and Soybean
Germany	Corn
Portugal	Corn
Argentina	Corn
Romania	Corn

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# **Safety Assessment of Transgenic Organisms**

OECD CONSENSUS DOCUMENTS

*Volume 2*



ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

## ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

The OECD is a unique forum where the governments of 30 democracies work together to address the economic, social and environmental challenges of globalisation. The OECD is also at the forefront of efforts to understand and to help governments respond to new developments and concerns, such as corporate governance, the information economy and the challenges of an ageing population. The Organisation provides a setting where governments can compare policy experiences, seek answers to common problems, identify good practice and work to co-ordinate domestic and international policies.

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*This book is published on the responsibility of the Working Group on Harmonisation of Regulatory Oversight in Biotechnology, which is a subsidiary group of the Chemicals Committee and Working Party on Chemicals, Pesticide and Biotechnology of the OECD.*

## FOREWORD

Genetically engineered crops (also known as transgenic crops) such as maize, soybean, rapeseed and cotton have been approved for commercial use in an increasing number of countries. During the period from 1996 to 2005, for example, there was more than fifty-fold increase in the area grown with transgenic crops worldwide, reaching 90 million hectares in 2005<sup>1</sup>. Such approvals usually follows a science-based risk/ safety assessment.

The environmental safety/ risks of a transgenic organism have been assessed based on the information on the characteristics of the host organism, the introduced traits, the environment into which the organism is introduced, the interaction between these, and the intended application. The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on identifying parts of this information, which could be commonly used in countries for environmental safety/ risk assessment to encourage information sharing and prevent duplication of effort among countries. Biosafety Consensus Documents are one of the major outputs of its work.

Biosafety Consensus Documents are intended to be a "snapshot" of current information on a specific host organism or trait, for use during regulatory assessments. They are not intended to be a comprehensive source of information on everything that is known about a specific host or trait; but they do address the key or core set of issues that member countries believe are relevant to risk/ safety assessment. This information is said to be mutually acceptable among member countries. To date, 25 Biosafety Consensus Documents have been published. They include documents which address the biology of crops, trees and micro-organisms as well as those which address specific traits which are used in transgenic crops.

This book is a compilation of those Biosafety Consensus Documents published before February 2006. It also includes two recently published texts: the first, entitled *An Introduction to the Biosafety Consensus Document of OECD's Working Group for Harmonisation in Biotechnology*, explains the purpose of the consensus documents and how they are relevant to risk/ safety assessment. It also describes the process by which the documents are drafted using a "lead country" approach. The second text is a *Points to Consider for Consensus Documents on the Biology of Cultivated Plants*. This is a structured checklist of "points to consider" for authors when drafting or for those evaluating a consensus document. Amongst other things, this text describes how each point is relevant to risk/ safety assessment.

This book offers ready access to those consensus documents which have been published thus far. As such, it should be of value to applicants for commercial uses of transgenic crops, regulators in national authorities as well as the wider scientific community. As each of the documents may be updated in the future as new knowledge becomes available, users of this book are encouraged to provide any information or opinions regarding the contents of this book or indeed, OECD's other harmonisation activities. If needed, a short pre-addressed questionnaire is attached at the end of this book that can be used to provide such comments.

The published Consensus Documents are also available individually from OECD's website (<http://www.oecd.org/biotrack>) at no cost.

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1. Clive James (2005), International Service for the Acquisition of Agri-biotech Applications (<http://www.isaaa.org/>)



## TABLE OF CONTENTS

CONSENSUS DOCUMENTS ON THE BIOLOGY OF TREES .....	9
Section 1 Eastern White Pine ( <i>Pinus strobus</i> L.).....	10
Section 2 European White Birch ( <i>Betula pendula</i> Roth).....	45
Section 3 Norway Spruce ( <i>Picea abies</i> (L.) Karst).....	75
Section 4 Poplar ( <i>Populus</i> L.).....	100
Section 5 Sitka Spruce ( <i>Picea Sitchensis</i> (Bong.) Carr.) .....	136
Section 6 Stone Fruits ( <i>Prunus</i> spp.).....	175
Section 7 White Spruce ( <i>Picea glauca</i> (Moench) Voss).....	204
CONSENSUS DOCUMENTS ON MICRO-ORGANISM.....	237
Section 1 Baculoviruses .....	238
Section 2 Pseudomonas.....	312
Section 3 <i>Acidithiobacillus</i> .....	394
LEAD COUNTRY AND PUBLISHED YEAR OF EACH CONSENSUS DOCUMENT .....	442
QUESTIONNAIRE TO RETURN TO THE OECD .....	443

## Tables

Table 3.1	Summary of successful crosses with <i>P. strobus</i> .....	15
Table 3.2	Species Interactions with Eastern White Pine .....	20
Table 3.3	The taxonomy and distribution of the 44 species in the genus <i>Betula</i> .....	46
Table 3.4	A summary of the results on seed germination in different crosses between <i>Betula</i> species .....	57
Table 3.5	Suggested classification, nomenclature and occurrence of <i>Populus</i> species and synonyms given by an earlier classification.....	104
Table 3.6	Nomenclature of naturally occurring <i>Populus Hybrids</i> .....	106
Table 3.7	Species cross compatibility with Sitka spruce.....	142
Table 3.8	Species Interactions with Sitka Spruce.....	148
Table 3.9	Stone fruit production, 1989-1999.....	176
Table 3.10	Interspecific hybrids with <i>Prunus persica</i> .....	190
Table 3.11	Hybrids of the sand cherries with other species .....	191
Table 3.12	<i>Prunus</i> species reported as hybrids between peach and peach species.....	192
Table 3.13	Species cross compatibility with White Spruce.....	216
Table 4.1	List of assigned baculovirus species .....	239
Table 4.2	Restriction maps of different nucleopolyhedroviruses and granuloviruses.....	244
<b>Bookmark not defined.</b>		
Table 4.3	Insect host transposons found in baculovirus .....	266
Table 4.4	Phylogeny and current classification of the pseudomonads.....	316
<b>Error! Bookmark not defined.</b>		
Table 4.5	Examples of plasmids responsible for the metabolism of organic compounds or resistance to heavy metals in fluorescent <i>Pseudomonas</i> species .....	319
Table 4.6	Examples of fluorescent species of <i>Pseudomonas</i> reported to have been used, or to have potential use, for bioremediation.....	327
Table 4.7	Examples of plasmids encoding for drug resistances in <i>P. aeruginosa</i> .....	338
Table 4.8	Range of plant species susceptible to infection with <i>P. syringae</i> .....	347
Table 4.9	Some toxins produced by phytopathogenic <i>Pseudomonas</i> spp. ....	348
Table 4.10	Phytopathogenic strains of <i>P. syringae</i> containing plasmids.....	352
<b>Error! Bookmark not defined.</b>		
Table 4.11	Examples of identification and detection techniques.....	356
Table 4.12	<i>Acidithiobacillus</i> : Characters used in classification .....	396
Table 4.13	Genes in <i>Acidithiobacillus ferrooxidans</i> .....	405
Table 4.14	Comparative % solubilisation of heavy metals in sludge.....	411
Table 4.15	Usage and environmental impacts of <i>Acidithiobacillus</i> .....	416
<b>Error! Bookmark not defined.</b>		

## Figures

Figure 3.1	The natural range of Eastern White Pine .....	12
Figure 3.2	The leaf, scale, seed, stem, buds and female and male catkins of <i>Betula pendula</i> .....	47
Figure 3.3	The natural distribution of <i>Betula pendula</i> .....	52
Figure 3.4	Crossability of <i>Populus</i> species .....	112
Figure 3.5	The natural range of Sitka spruce .....	138
Figure 3.6	The natural range of White Spruce .....	208
Figure 3.7	The reproductive cycle of White Spruce .....	210
Figure 4.1	Morphological characteristics of nucleopolyhedroviruses and granuloviruses ... ..	241



*Part 3*

**CONSENSUS DOCUMENTS ON THE BIOLOGY OF TREES**

## SECTION 1 EASTERN WHITE PINE (*PINUS STROBUS* L.)

### 1. General Information

This consensus document addresses the biology of Eastern White Pine (*Pinus strobus* L.), referred to hereafter simply as Eastern White Pine (*pin blanc* in French Canada). Eastern White Pine is one of the most valuable tree species in eastern North America where its easily machined, uniform-textured wood is unsurpassed for doors, windows, panelling, mouldings and cabinet work (Mullins and McKnight, 1981; Farrar, 1995). The species played a major role in the settlement and economic development of New England and the Atlantic Provinces as England reserved all large Eastern White Pine suitable for masts under the "Broad Arrow" policy, starting in the late 1600's (Johnson, 1986). Eastern White Pine also responds well to nursery culture and is commonly used for reforestation, urban forestry and Christmas tree plantations.

The general biology of Eastern White Pine is described in the context of the species' role in natural forests and its domestication in planted stands. Taxonomic and evolutionary relationships with other *Pinus* species are described. Reproductive biology is described with a focus on aspects of mating system, gene flow, seed production and natural stand establishment. The current knowledge of genetic variation within the species is reviewed, highlighting the importance of geographic variation patterns and the potential for improvement by means of recurrent selection breeding strategies. The tremendous biological diversity and the complexity of ecological interactions with higher and lower flora and fauna are discussed. While Eastern White Pine has been commonly planted within its natural range, the extent of reforestation has been limited by susceptibility to white pine weevil (*Pissodes strobi*) and blister rust (*Cronartium ribicola*). Domestication and operational breeding activities are also reviewed. Crossing with other related white pine species offers some promise of producing hybrids with increased resistance to both the weevil and blister rust. While white pine reforestation is currently based on seed propagation, vegetative propagation techniques are available and research continues into regeneration from somatic embryos.

Canada was the lead country in preparation of this document. It is intended for use by regulatory authorities and others who have responsibility for making assessments of transgenic plants proposed for commercialisation, and by those who are actively involved with genetic improvement and intensive management of this species.

### 2. Taxonomy and Natural Distribution

#### A. Taxonomy and nomenclature

The genus *Pinus* L. (family Pinaceae) is widely distributed throughout the Northern Hemisphere, from the arctic circle south to Guatemala, the West Indies, North Africa and Indonesia, with as many as 100 species being recognised (Krüssmann, 1985). The genus was first classified on evolutionary characteristics by Shaw (1914), and taxonomists have since followed his general separation of the genus into two groups: *Haploxylon* Koehne, and *Diploxylon* Koehne; commonly called the "soft" (or "white") and "hard" pines, respectively, based on the presence of one or two vascular bundles in the leaves. Shaw's original subdivision of these groups has been reworked by different authorities (e.g., Pilger, 1926; Duffield, 1952;

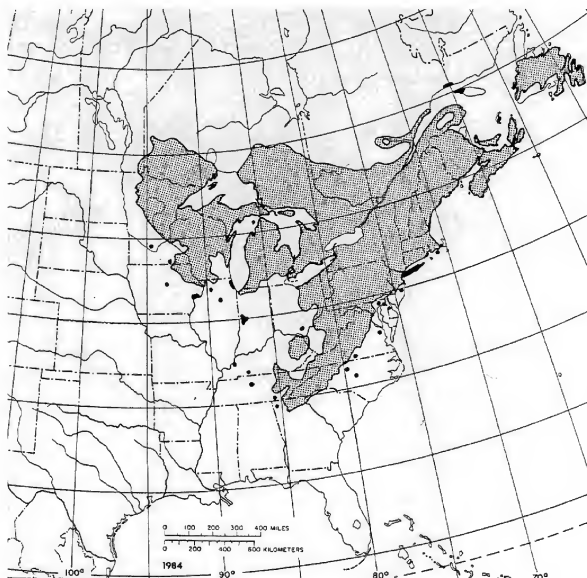
de Ferré, 1965; Landry, 1974b, 1978), but botanists in recent years have generally recognised the classification described by Little and Critchfield (1969, 1986), who place Eastern White Pine, *Pinus strobus* L., within the subgenus *Strobus* Lemm. (equivalent to subgenus *Haploxylon*), section *Strobus*, subsection *Strobi* Loud. Also known as northern pine and, in parts of Europe, as Weymouth pine, after Lord Weymouth, the species nomenclature has remained virtually undisputed since the publication of the *Species Plantarum* (Linné, 1753), although Provancher later referred to it as *Pinus alba Canadensis* Prov. (Landry, 1974a).

Several horticultural forms have been named, although none are currently recognised with varietal status (Krüssmann, 1985). Only one variety has been commonly described, *Pinus strobus* L. var. *chiapensis* Martinez, the Chiapas white pine, occurring in the mountains of southern Mexico and Guatemala. While similar morphologically, it is physiologically quite different (Wright, 1970) and now generally recognised as a separate species, *Pinus chiapensis* (Martinez) Andresen (Griffiths, 1994; Perry, 1991).

## **B. Natural distribution**

Eastern White Pine has the largest range of any North American member of subgenus *Strobus*, and is the only species in the subgenus occurring on the eastern side of the continent. It extends from Newfoundland and Quebec, west to central Ontario and south-eastern Manitoba, south to Minnesota, north-eastern Iowa, northern Illinois, north-western Indiana, Ohio, Pennsylvania, and New Jersey, and south in the Appalachian Mountains to western North Carolina, northern Georgia, and Tennessee. Overall, the species spans a north-south range of over 1900 km, and about the same distance inland from the Atlantic coast (Critchfield and Little, 1966; Mirov, 1967; Wendel and Smith, 1990). The natural range of Eastern White Pine is illustrated in the map given in Figure 3.1.

Figure 3.1 The natural range of Eastern White Pine



Source : Wendel and Smith, 1990

### C. Evolution and migrational history

Conifers probably originated around the periphery of the north Pacific basin (Li, 1953). Fossil records indicate that divergence of modern genera in Pinaceae occurred some 135 million years ago during the late Jurassic or early Cretaceous period (Florin, 1963), and *Pinus* is believed to be the oldest genus in the family (Miller, C.N. 1976, 1988). Opinion on whether the first pines were of subgenus *Strobus* or *Pinus* remains mixed, and the centre of origin of pines is uncertain. Millar and Kinloch (1991) describe the rapid spread of pines over dry, temperate paleolatitudes during the Cretaceous period, prior to the separation of North America from Europe, during which all sections and subsections appear to have originated. In the early Tertiary period, global climate changes favoured the spread of angiosperms, which adapted to the hot, humid conditions. These climate changes fragmented and displaced the pines into drier refugia at upper and lower latitudes, and scattered refugia at mid-latitudes, creating secondary centres of origin. Ancestors

of *P. strobus* and *P. monticola* were isolated in northern refugia from other species in section *Strobi* that were isolated in the south. The warm, tropical conditions changed rapidly at the end of the Eocene epoch and pines became re-established at middle latitudes. These abrupt changes in climate had drastic impacts on the gene structure of genetic variation of forest populations, with isolated populations continuing their short-term evolution (Critchfield, 1984).

### 3. Reproductive Biology

#### A. Reproductive development

Eastern White Pine is monoecious. Production of female strobili occurs as early as 4 years (Buckingham, 1963) while pollen production may not start for 10 to 20 years (Wright, 1970). As in other pines, development of the reproductive structures follows a 3-year cycle. Pollination occurs in the spring of the second year, with fertilisation delayed until the following spring, and seeds maturing in the fall of the third year (Owens and Blake, 1985). No other conifer genus has had its reproductive cycle described more often or more thoroughly, and Eastern White Pine was among the first pines to be studied in detail (Ferguson, 1901, 1904). Reproductive buds begin as axillary bud primordia within a complex long-shoot bud, consisting of a series of cataphylls initiated throughout the growing season. Many of the cataphylls support an axillary apex that first initiates a series of bud scales, then differentiates into a short (fascicular) shoot, seed or pollen cone, or lateral long shoot bud. Those axillary buds initiated at the base of the long shoot bud in the spring or early summer will differentiate into short-shoot or pollen-cone buds. Subsequent axillary buds differentiate only into short shoots. The distal axillary buds remain undetermined through winter dormancy of the long shoot bud, differentiating immediately the following spring into lateral long shoot or seed cone buds (Owston, 1969; Owens and Molder, 1977). While seed-cones generally develop on vigorous shoots in the upper portion of the crown, distribution of reproductive structures is often extremely variable.

Pollen development and meiosis does not occur until the spring of the second year as pollen cones resume their development. The ripening strobili turn light brown before releasing their pollen over a 1-week period. The seed cones also resume development in the spring, and are visible at the distal end of elongating long shoots. The developmental morphology of reproductive structures was well documented with colour photographs by Ho (1991). Wind-borne pollen grains landing on the receptive seed cone pass between the bracts and sift down to the surface of the micropylar arms, entering the micropyle by means of a pollen drop. The pollen germinates, but becomes dormant before the male gametes form (Owens and Molder, 1977). Fertilisation occurs about 13 months after pollination. Simple polyembryony in Eastern White Pine results from the fertilisation of 2 to 3 archegonia in each megagametophyte. The seed cones mature and seeds are dispersed in August or September of the same year (Krugman and Jenkinson, 1974).

#### B. Mating system and gene flow

Eastern White Pine is a wind-pollinated, monoecious species, and outcrossing is by far the most prevalent mating system, although there are relatively few detailed studies. Isozyme studies of populations in Québec indicated a high rate of outcrossing, with most loci in Hardy-Weinberg equilibrium (Beaulieu and Simon, 1994, 1995).

Gene flow in *Pinus* is mediated by very small pollen grains, 40–60  $\mu\text{m}$  at their widest point (Eisenhut, 1961), whose two air sacs and low density make them well-adapted for aerial transport (Di-Giovanni and Kevan, 1991). Various studies of pollen dispersal in conifers indicate that over 90% of the pollen comes to rest less than 100 m from the source (Wright, 1976). Nevertheless, conifer pollen may remain viable for several days and a substantial quantity may travel great distances (Lindgren *et al.*, 1995; Lindgren and Lindgren, 1996). Gregory (1973) cites reports that pollen of *Pinus* and *Picea* may travel as far as 600 to

1 000 km, and several authors have concluded that isolation distances of less than 1 km often have little impact on contamination rates in conifer seed orchards (see review by Di-Giovanni and Kevan, 1991).

### C. Seed production

Eastern White Pine normally begins seed production at 5 to 10 years of age (Fowells, 1965; Sargent, 1965), although little pollen is produced during the early years of flowering (Wendel and Smith, 1990). The interval between heavy seed crops is usually 3 to 10 years (Krugman and Jenkinson, 1974; Wendel and Smith, 1990), becoming less frequent as trees become over mature (Horton and Bedell, 1960). A study in Germany recorded seed production as high as 73 kg/ha in a 90-year-old stand (Messer, 1956), while in Maine, a stand considered to be intermediate in density with a basal area of 28 m<sup>2</sup>/ha produced over 4.4 million seeds per hectare in a "bumper" year (Grabner, 1970). This corresponds to 69kg/ha seed.

Initiation of seed dispersal is weather and site dependent, and may be delayed by cool, moist weather. Most of the seeds are dispersed in the fall during a 4 to 8 week period (Horton and Bedell, 1960; Grabner, 1970). The seeds are mature when cone moisture content decreases below 200% on a dry-weight basis, but cone specific gravity is not a reliable indicator of maturity (Barnett, 1988). A short "artificial ripening" period can increase yield and quality of seed from immature cones (Bonner, 1986, 1991; Barnett, 1988).

The seeds are winged and dispersal distances depend greatly on local and prevailing wind patterns (Rudis *et al.*, 1978). The seeds may travel more than 60 m within a closed stand, and over 200 m in the open (Wilson and McQuilkin, 1963), although most of the seed will fall within a distance equivalent to the height of the seed tree (Horton and Bedell, 1960). The seeds themselves are smaller than those of most other "soft" pines, but similar to those of *P. monticola*, with average cleaned seed weight of about 17 g/1000 seeds (Krugman and Jenkinson, 1974).

### D. Natural regeneration

Eastern White Pine seeds exhibit varying degrees of embryo dormancy that may be broken by exposure to low temperatures under moist conditions, *i.e.*, cold stratification (Krugman and Jenkinson, 1974; Nelson *et al.*, 1980; Mittal *et al.*, 1987; Downie and Bergsten, 1991). The recommended treatment for nursery sowing is stratification for 60 days at 1 to 5° C (Krugman and Jenkinson, 1974). Under natural conditions, over-winter stratification on the forest floor breaks seed dormancy and germination of most seeds occurs in late spring of the following year (Stiell, 1985).

Germination is epigeal. Moist mineral soil, polytrichum moss, and shortgrass cover of light to medium density are favourable seedbeds. Establishment on less favourable seedbeds, such as pine litter and lichen, will occur under partial shade and/or surface. Shelterwood harvesting systems provide good protection during initial establishment with sufficient light for subsequent growth of young stands (Wilson and McQuilkin, 1963; Corbett, 1994). Optimum conditions are provided when moist mineral seedbeds have greater than 20% of full sun, but where partial shade reduces surface temperatures and provides better moisture conditions (Lancaster and Leak, 1978). Low seedling densities are associated with competition from broad-leaved shrubs, herbaceous vegetation, tolerant conifer species and feather mosses (Carleton *et al.*, 1996). White pine regeneration is usually associated with its proportion in the overstorey (Kittredge and Ashton, 1990), and under old-growth conditions is likely to become at least partially uneven-aged and self replacing, facilitated by local disturbances and continuous recruitment (Quinby, 1991; Ziegler, 1995). Older trees have increased their ability to recover from long periods of suppression (Abrams and Orwig, 1996).

### E. Vegetative reproduction in nature

Eastern White Pine does not regenerate vegetatively under natural conditions (Wendel and Smith, 1990).

### 4. Crosses

Other members of subgenus *Strobos* do not occur within the natural range of Eastern White Pine, and introgressive hybridisation does not occur. Most artificial crosses among North American members of subsection *Strobi* have been successful, the exception being those involving *Pinus lambertiana* Dougl. (Critchfield, 1986; Critchfield and Kinloch, 1986). Successful crosses involving Eastern White Pine are summarised in Table 3.1. Only two of these hybrids have been widely field tested, including that with *P. monticola* Dougl. and its reciprocal, and with *P. wallichiana* A.B. Jackson (formerly *P. griffithii* McClell.) and its reciprocal (Kriebel, 1983). No successful hybrid crosses have been reported with species in other sections of *Pinus* (Critchfield, 1975).

Table 3.1 Summary of successful crosses with *P. strobus*

Species	Origin	References
<i>P. monticola</i> Dougl. ex D.Don.	western US and Canada	Wright, 1959, 1970; Kriebel, 1972b
<i>P. wallichiana</i> A.B. Jackson (syn <i>P. griffithii</i> McClell., <i>P. excelsa</i> Wallich ex D.Don), hybrid = <i>P. schwenkeri</i> Fitschen	Himalayas	Wright, 1959, 1970; Kriebel, 1972b; Garrett, 1979; Zsuffa, 1979b; Blada, 1992
<i>P. ayacahuite</i> Ehrenb.	Mexico, Guatemala	Johnson and Heimbürger, 1946; Wright, 1959; Garrett, 1979
<i>P. parviflora</i> Sieb. and Zucc. hybrid = <i>P. hunnewellii</i> A.G. Johnson	Japan	Johnson, 1952; Wright, 1959
<i>P. peuce</i> Griseb.	S.E. Europe	Fowler and Heimbürger, 1958; Radu, 1976; Santamour and Zinkel, 1978
<i>P. flexilis</i> James (only one parent successful, may in fact be <i>P. strobiformis</i> Engelm.)	western US and Canada	Wright, 1959; Kriebel, 1972a

When hybrids are made successfully, they sometimes display hybrid vigour and out-perform the parent species (Wright, 1970; Kriebel, 1983). However, more important than increased vigour, hybrids with *P. wallichiana*, *P. peuce*, and *P. parviflora* have demonstrated potential resistance to blister rust (Heimbürger, 1962, 1972; Patton, 1966; Zsuffa, 1979a), and those with *P. peuce* and *P. monticola* may be less susceptible to weevil attack (Heimbürger and Sullivan, 1972a, b). While hybridity barriers within the hard pines are generally associated with pollen tube incompatibility, crossability barriers among the white pines are more often the result of embryo inviability (Kriebel, 1972a; Shafer and Kriebel, 1974).

### 5. Genetics

#### A. Cytology

Vegetative cells are normally diploid, with  $2n = 24$  chromosomes (Saylor, 1983). Saylor and Smith (1966) reported that 4% of cells displayed meiotic irregularities such as precocious disjunction, lagging chromosomes, and inversion bridges.

## **B. Genetic variation**

### ***Population-level variability***

While seed source testing of Eastern White Pine began in the United States in 1937 (Pauley *et al.*, 1955), provenance tests with range-wide sampling did not begin until the mid-1950's. Around this time, the USDA Forest Service initiated a large provenance test, in which 30 seed collections representing all parts of the natural range were established by co-operators in 13 test plantations in the United States and 2 in Ontario (Sluder, 1963; Wright *et al.*, 1963; Funk, 1965; Fowler and Heinburger, 1969b; King and Nienstaedt, 1969; Genys, 1977). Shortly after, another provenance test involving more seedlots on fewer test sites was started by the University of Maryland (Genys, 1968; Genys *et al.*, 1978). Encouraging early results from these tests, indicating the superiority of sources from the South Appalachians, led to intensive testing of these sources under the leadership of Michigan State University (Roth and Carson, 1976; Wendel and Cech, 1976; Wright *et al.*, 1976; Gall and Thor, 1977).

While correlations with latitude have sometimes been noted on a range-wide basis (Genys, 1987, 1991), relative differences in height and diameter between northern and southern sources diminish somewhat with age (Demeritt and Kettlewood, 1976; Demeritt and Garrett, 1996). Clinal patterns are often less distinct over shorter distances with the presence of non-clinal adapted ecotypes (Genys, 1968; Garrett *et al.*, 1973; Thor, 1975; des Bordes and Thor, 1979; Funk, 1979). In Nebraska, seed sources from the southern Appalachians demonstrated correlations with latitude for needle length and reproductive phenology, a weak geographic pattern for variation in height, and none for survival (Sprackling and Read, 1976; Van Haverbeke, 1988). Ryu and Eckert (1983) investigated the genetic structure of 27 of these provenances for eight foliar enzymes coded by 12 loci and found four clusters of provenances, three of which may be representative of populations adapted to differing geographic and climatic conditions. The results of this study support the indication of ecotypic variation among three provenances in the southern Appalachians for growth performance and physiological variables, and suggest that these areas may have been isolated refugia during glaciation. Elsewhere in the northern part of the range, sources from the Atlantic coast outperformed those from further inland, while some exceptional sources originated from as far south as Georgia and Tennessee (Zsuffa, 1975; Abubaker and Zsuffa, 1991).

Southern provenances have heavier seeds (Genys, 1968), require longer periods of stratification before germinating (Fowler and Dwight, 1964; Graber, 1965) and longer chilling periods to break bud dormancy (Mergen, 1963), set bud later (Santamour, 1960) and are less cold hardy (Maronek and Flint, 1974). Wood specific gravity was negatively correlated with height and diameter, but differences among sources were small (Lee, 1974; Gilmore and Jokela, 1978; Olson *et al.*, 1981). No variation could be detected for foliar monoterpene content, and no geographic pattern was evident for variation in cortical monoterpenes (Gilmore and Jokela, 1979).

Provenance tests have shown some variation in susceptibility to white pine weevil, but give little indication that resistant populations can be identified (Garrett, 1972, 1973; Connola and Beinkafner, 1976; Wilkinson, 1983b). Selective thinning of susceptible parents (dominant "wolf" trees) from a stand can increase the level of resistance in the progeny generation, and taller families tend to be more weevil resistant (Ledig and Smith, 1981). Although there is ample evidence of genetic control of susceptibility to weevil, the actual mechanism(s) of resistance remains uncertain.

### ***Individual-level variability***

While variation among provenances is important in determining the risks and benefits of transferring seed sources, genetic improvement from mass selection relies primarily on variation within-populations as the source of genetic gains. The partitioning of genetic variance among and within populations is greatly

influenced by the range of adaptive variation sampled by the tested provenances and the age at which the test material is assessed. Range-wide and regional studies have typically demonstrated strong heritabilities, sufficient to predict moderate to high genetic gains, although heritability tends to be lower for older material (Thor, 1975; Adams and Jolly, 1978; des Bordes and Thor, 1979; Olson *et al.*, 1981). Hierarchical sampling of populations over a more limited range in Québec and Ontario showed that population differences were greatest for allozyme markers, where 98% of the variation was within populations (Beaulieu *et al.*, 1996). Growth traits, on the other hand, demonstrated variation within stands to be about half as great as that among populations (Li, P. *et al.*, 1997). Individual heritability for height declined from 0.547 at age 4 in the nursery, to 0.187 at age 10 in the field (Beaulieu *et al.*, 1996). In an incomplete diallele cross experiment among individuals of a local provenance, Kriebel *et al.* (1972) found that narrow-sense heritability for height growth declined from 0.59 at age 1 to 0.16 at age 3, and that while dominance effects were small, maternal effects were rather large. By age 13, it was still possible to achieve substantial gains by family selection (Kriebel, 1978).

Significant genotype-environment interactions have been reported in Eastern White Pine, but the magnitude of the interaction variance is generally low (less than 2%). Genetic correlations between sites tend to be high, indicating that family ranks are stable across sites (des Bordes and Thor, 1979; Beaulieu *et al.*, 1996; Demeritt and Garrett, 1996).

The search for weevil-resistance has always been a driving force behind genetic testing in Eastern White Pine (Pauley *et al.*, 1955; Wright and Gabriel, 1959). Early studies indicated that selection for weevil resistance might be done indirectly by assessment of bark thickness (Kriebel, 1954; Gerhold, 1962, 1966) and/or leader morphology (Stroh, 1964, 1965), but when these are corrected for tree size, they appear to be of little value for effective selection (Wilkinson, 1983a, 1984). Other studies have identified that concentrations of various cortical oleoresin compounds are correlated with weevil susceptibility, but even these criteria leave much of the variation in weevil susceptibility unexplained (van Buijtenen and Santamour, 1972; Santamour and Zinkel, 1976, 1978; Bridgen *et al.*, 1979; Wilkinson, 1979, 1980, 1984, 1985).

### C. Inbreeding depression and genetic load

Eastern White Pine is an outcrossing species that carries a fairly heavy load of deleterious recessive genes. Individuals are generally self-compatible, so that this genetic load is revealed by self-fertilisation (Fowler, 1965a; Fowler and Heimbürger, 1969a). Although there is no reduction in numbers of filled seeds after selfing (Fowler, 1965b), selfed seedlings may be stunted, slow growing, chlorophyll-deficient and deformed (Johnson, 1945; Patton and Riker, 1958a; Fowler, 1965b). Simple polyembryony in Eastern White Pine results from 2 to 3 archegonia in each megagametophyte. As only one embryo normally germinates from the mature seed, it is likely that competition during seed development eliminates many weaker embryos, including those resulting from self-fertilisation (Willson and Burley, 1983). An isozyme study of populations in Quebec demonstrated a high outcrossing rate, with few loci deviating from Hardy-Weinberg equilibrium (Beaulieu and Simon, 1995). This study found evidence of family structure, with greater inbreeding in the filial than in the parental population, although few of the inbred genotypes were expected to reach reproductive age, due to natural selection.

### D. Breeding programs

Eastern White Pine has been a candidate for tree breeding efforts throughout its native range. In the northern part of its range, throughout eastern Canada, the north-eastern US and the Lake States, planting programs have been limited by susceptibility to weevil and rust, so that seed orchards exist throughout this region (Zsuffa, 1985, 1986; Garrett, 1986; Miller, 1987; Eckert and Kuser, 1988; Lamontagne, 1992; Nielsen *et al.*, 1995; Smith *et al.*, 1997; *pers. comm.* R. Stine, Minnesota Tree Improvement Cooperative)

and, the level of effort reflects the restricted size of planting programmes. Pests are less of a problem for breeding programs in the Central States, where selection and hybrid breeding can focus on vigour (Kriebel, 1983). Outside of the natural range in Europe, selection within southern Appalachian provenances and crossing with other white pines, such as *Pinus wallichiana*, are used to develop fast-growing, rust-resistant hybrids (Kriebel, 1983).

Most seed orchards currently in production were established by grafting cuttings from plus-trees, and their establishment in cultivated field environments. Grafting success is usually very high. Flowering in field orchards can be enhanced by means of cultural treatments such as fertilisation (Hocker, 1962; Stephens, 1964). Flowering of young white pine grafts can also be stimulated by means of various cultural treatments, particularly those involving gibberellin A<sub>4</sub>/7, and this has facilitated the turnover of breeding cycles (Ho and Schnckenburger, 1992; Ho and Eng, 1995).

#### **E. Conservation of genetic resources**

Domestication of a key species such as Eastern White Pine can influence diversity of genetic resources (1) indirectly, by the method of seed collection, extraction, and storage, and by nursery and plantation culture; and (2) directly, by intentional selection to increase the frequency of genes for desirable traits (Morgenstern, 1996). The inadvertent loss of genes by natural processes and human activity can have negative consequences on the adaptability of populations and the potential for future gains from breeding.

A long history of exploitation has resulted in white pine forest fragmentation and reduction of population sizes, particularly at the northern limits of the species range (Buchert, 1994; Buchert *et al.*, 1997). Throughout most of the range of white pine, *in situ* conservation of genetic resources is practised by protection of ecological reserves, special areas, and parks (Pollard, 1995), and integrated with domestication activities that control the movement of seed, active management of existing stands to maintain biological diversity, and protection of isolated, small populations (Mosseler, 1995; Nieman *et al.*, 1995).

*Ex situ* conservation, by cryopreservation of germplasm, by off-site maintenance of populations in arboreta, seed orchards and clone banks, and by multi-population breeding strategies (Eriksson *et al.*, 1993; Namkoong, 1995), has been practised to a much lesser extent, although many provenances and families of Eastern White Pine are now represented in field tests and seed bank collections (Plourde *et al.*, 1995). Such "active" forms of gene management must be accelerated in preparation for response to rapid environmental and climate changes (Ledig and Kitzmiller, 1992).

#### **6. Ecology and Associated Species**

Much of the information in this section originates from the excellent monograph on silvics of the species by Wilson and McQuilkin (1963). Other citations are given when appropriate when specific information is attributable to other sources.

##### **A. Habitat**

###### *Climate*

Eastern White Pine's natural range is cool and humid. July average temperatures are between 18 to 25° C, and annual precipitation varies from about 510 mm in northern Minnesota to 2030 mm in north-western Georgia, with at least half occurring between April and November. Average snowfall varies from less than 15 cm in the southern portion of the range to over 250 cm in the northeast (Wendel and Smith, 1990). There is a surplus of moisture in all seasons.

### Soils and site type

Eastern White Pine grows on a wide variety of soils throughout its range, from dry sands and rocky ridges, to sphagnum bogs, although it grows best on moist sandy or loamy soils. Soils within the range are derived from granites, gneisses, schists, sandstones, and, to a lesser extent, phyllites, slates, shales and limestones. Eastern White Pine competes best on medium-textured, well-drained soils of moderate site quality, with surface pH between 4.0 and 7.5, and which are not sufficiently rich to support strong hardwood competition, or where competition is reduced during the establishment period, such as on old fields, burnt or blow-down areas (Horton and Bedell, 1960; Mader, 1986).

In the northeast portion of the range, Eastern White Pine generally occurs below 450 m above sea level, whereas in Pennsylvania, elevations vary between 150 and 600 m. In the southern Appalachians, stands generally occurs between 370 and 1070 m. Except in Pennsylvania and the southern Appalachians where stands are found on northerly aspects or in the shelter of stream bottoms. White pine sites are not generally restricted by slope or aspect.

### B. Synecology and associated species

Eastern White Pine may form pure stands or occur as a major stand component of several stand types in association with other conifers and hardwoods such as: red pine (*Pinus resinosa*), balsam fir (*Abies balsamea*), black spruce (*Picea mariana*), White Spruce (*P. glauca*), red oak (*Quercus rubra*), sugar maple (*Acer saccharum*), red maple (*Acer rubrum*), hemlock (*Tsuga canadensis*), and chestnut oak (*Quercus prinus*). Eastern White Pine may also be found as a lesser stand component with jack pine (*Pinus banksiana*), pitch pine (*P. rigida*), shortleaf pine (*P. echinata*), sweet birch (*Betula lenta*), trembling aspen (*Populus tremuloides*), large-tooth aspen (*P. grandidentata*), black cherry (*Prunus serotina*), black oak (*Quercus velutina*), white oak (*Quercus alba*), and various hickories (*Carya* spp.) (Horton and Bedell, 1960; Eyre, 1980). The occurrence of associations depends on both site conditions and history of disturbance (Stiell, 1985).

Pure stands of Eastern White Pine usually support sparse cover of understory vegetation, but many species may be found under mixed stands, particularly those associated with hardwood associates. On drier sites, ground vegetation may consist of one or more species of blueberries (*Vaccinium* spp.), teaberry (*Gaultheria procumbens*), dwarf bush-honeysuckle (*Diervilla lonicera*), sweetfern (*Comptonia peregrina*) bracken fern (*Pteridium aquilinum*), clubmoss (*Lycopodium* spp.), and broom sedge (*Andropogon virginicus*). Richer, moist sites will often support ground cover of woodsorrels (*Oxalis* spp.), partridgeberry (*Mitchella repens*), wild sarsaparilla (*Aralia nudicaulis*), jack-in-the-pulpit (*Arisaema* spp.), and hay-scented fern (*Dennstaedtia punctilobula*). Intermediate sites may have varying amounts of the above species, together with dogwoods (*Cornus* spp.) and false lily-of-the-valley (*Maianthemum canadense*).

### C. Competition and stand structure

Eastern White Pine is distributed over a larger area than any other North American white pine, and has demonstrated its capacity to grow and compete under a wide variety of environmental conditions (Stiell, 1978, 1985). While it is a long-lived successional species and may be a component of climax forest types, it is also well-known as a pioneering species on old fields in New England. Eastern White Pine is considered intermediate in its tolerance to shade, somewhat less tolerant than eastern spruces and more tolerant than its pine associates (Daniel *et al.*, 1979). Vegetative competition for light and soil moisture is critical during seedling establishment, and remains important well into the life of the stand. Sites that have a high capability for productivity for pine tend to have greater competition. Competition problems are most severe on heavier, moist, rich soils, where Eastern White Pine will perform well, only if natural disturbance, such as fire, or silvicultural site treatments allow the pine to become established well ahead of

the hardwoods that normally occupy such sites (Horton and Bedell, 1960; Little *et al.*, 1973; Stiehl, 1985; Chapeskie *et al.*, 1989).

#### D. Ecosystem dynamics

Several abiotic factors also interact with Eastern White Pine in forest ecosystems. While older trees have thick, heat-resistant bark, the thinner bark on exposed roots and younger stems is sensitive to fire. Even light fires can have a detrimental impact on seed supply, but may also reduce hardwood competition and leave a seedbed that is more conducive to the establishment of new germinants. Frost heaving can cause severe damage, particularly to container seedlings planted on finer-textured soils. Eastern White Pine is relatively wind firm, but may suffer storm breakage if the stand has been recently thinned. While it is widely held that Eastern White Pine is sensitive to ozone and sulphur dioxide pollution (Gerhold, 1977), recent data in the literature are somewhat contradictory and suggest that injury and growth losses may be strongly genotype and site dependant (Houston and Stairs, 1973; Genys and Heggstad, 1978, 1983; Townsend and Dochinger, 1982; Usher and Williams, 1982; Yang *et al.*, 1982, 1983; Eberhardt *et al.*, 1988; Rezabek *et al.*, 1989; Bartholomay *et al.*, 1997; Hogsett *et al.*, 1997).

The following table 3.2 shows species interactions with Eastern White Pine.

**Table 3.2 Species Interactions with Eastern White Pine**

Insects	
Common name	Agent
White pine weevil [Sullivan, 1961; Sun and Nigam, 1972; Sunandram <i>et al.</i> , 1972; Berry and Steill, 1976; Sunandram, 1977; Stiehl, 1979; Martineau, 1984; deGroot, 1985; Drooz, 1985; Gross, 1985a; Wallace and Sullivan, 1985; Stiehl and Berry, 1985; Diamond and Bradbury, 1992; Katovich and Morse, 1992; Mielke, 1993; Humble <i>et al.</i> , 1994; de Groot and Zylstra, 1996]	<i>Pissodes strobi</i> The most serious economic insect pest of white pine. Larvae tunnel down the inner bark of the shoot, killing the leaders.
Sawfly [Houseweart and Knight, 1986]	<i>Diprion similis</i> . Foliage damage
Pine false webworm	<i>Acantholyda erythrocephala</i> Foliage damage
White pine sawfly	<i>Neodiprion pinetum</i> Foliage damage
Jack pine budworm	<i>Choristoneura pinus</i> (when growing near jack pine) Foliage damage
Eastern pine shoot borer	<i>Eucosma gloriosa</i> Growing shoot damage
European pine shoot moth	<i>Rhyacionia buoliana</i> Growing shoot damage
Pine leaf adelgid	<i>Pineus pinifoliae</i> (when growing near red or black spruce ) Causes shoot damage
White pine aphids	<i>Cinaria strobi</i> Can cause mortality in young trees
Seedling debarking weevil [Houseweart and Knight, 1986; Pendrel, 1990]	<i>Hylobius congener</i> Can cause seedling mortality
Warren's collar weevil	<i>H. warreni</i> Damages roots
Pine root collar weevil	<i>H. radialis</i> Damages roots
Pales weevil	<i>H. pales</i> Damages roots
Mound ants	<i>Formica</i> sp. Damages roots
Zimmerman pine moth	<i>Dioryctria zimmermani</i> . Damages sapling stems
Fir coneworm	<i>D. abietivorelle</i> reduces seed production
White pine cone beetle	<i>Conophthorus coniperda</i> reduces seed production
White pine cone borer [Wilson, 1977; Martineau, 1984; Rose and Lindquist, 1984; Syme, 1985]	<i>Eucosma toculionana</i> reduces seed production

Fungi	
Disease	Agent
White pine blister rust [Patton, 1961; Van Arsdel, 1961; Charlton, 1963; Gremmen and Kam, 1970; Anderson, 1973; Lehrer, 1982; Lavalée, 1974, 1986; Robbins, 1984; Gross, 1985b; Stiell, 1985; Ostrofsky <i>et al.</i> , 1988; Merrill, 1991; Katovich and Mielke, 1993; Myren <i>et al.</i> , 1994; Liebhold <i>et al.</i> , 1995; Berube, 1996; Bowling and Niznawske, 1996; Hummer, 1997; La and Yi, 1976; Yokota and Uozumi, 1976; Stephan and Hyun, 1983]	<i>Cronartium ribicola</i> . the most serious fungal disease of white pine. Has alternate host from the <i>Ribes</i> species as well as <i>Pedicularis</i> and <i>Castilleja</i> species.. Eradication of <i>Ribes</i> near white pine nurseries is a common control practice.
"Damping off" of emerging seedlings [Peterson, 1975]	<i>Fusarium</i> spp. <i>Pythium</i> spp. <i>Rhizoctonia</i> spp. <i>Phytophthora</i> spp. <i>Cylindrocladium</i> spp.
Cytospora dieback	<i>Valsa</i> spp. disease of young seedlings
Tip blight	<i>Sphaeropsis sapinea</i> disease of young seedlings
Snow blight	<i>Phacidium infestans</i> disease of young seedlings
Rhizinia root rot	<i>Rhizinia undulata</i> disease of young seedlings
Needle casts	<i>Lophodermium</i> spp. <i>Hypoderma</i> spp. <i>Cytospora</i> spp.
Brown spot needle blight	<i>Mycosphaerella dearnessii</i> Disease of foliage
Sooty mold	<i>Catenuloxylum semiovatum</i> Disease of foliage
Scleroderris canker	<i>Gremmeniella abietina</i> Disease of stems
White pine root decline [Hodges, 1986]	<i>Verticicladiella procera</i> Root disease
Armillaria root rot Belt fungus Tomentosus root rot Brown cubical root rot Black root stain [Syme, 1985; Hodges, 1986 and Myren <i>et al.</i> , 1994]	<i>Armillaria mellea</i> complex <i>Fomitopsis pinicola</i> <i>Inonotus tomentosus</i> <i>Heterobasidium annosum</i> <i>Verticicladiella</i> spp.
Animals	
Common name	Agent
Moose	<i>Alces alces</i> use pine stands for cover
White-tailed deer	<i>Odocoileus virginianus</i>
Porcupine	<i>Erethizon dorsatum</i> may feed on bark
Snowshoe hares [Radvanyi, 1987; Bergerson and Tardiff, 1988]	<i>Lepus americanus</i> commonly feed on bark and buds of young trees
Eastern cottontail rabbit	<i>Sylvilagus floridanus</i> commonly feed on bark and buds of young trees
Red squirrel [Syme, 1985]	<i>tamiasciurus hudsonicus</i> damages shoots in removing cones
Seed-eating birds	Many bird species commonly eat large quantities of seed

### E. Symbiotic Relationships - Mycorrhizae

Field data indicate that ectomycorrhizae formed by *Pisolithus tinctorius* increase survival and growth of *P. strobus* and other southern pine species better than natural ectomycorrhizae on routine reforestation sites in the southern U.S (Marx *et al.*, 1977). In Canada *Laccaria* sp., *Hebeloma* sp., *Tuber* sp. and *Telephora terrestris* form ectomycorrhizas with *P. strobus* seedlings grown in pot cultures, while *Phialophora finlandia*, an unidentified ascomycetous "red-type" fungus, and the E-strain form ectomycorrhizas (Schelkle *et al.*, 1996; Ursic and Peterson, 1997).

Some ectomycorrhizal fungi can suppress root-rotting pathogens of conifers. A study of natural mycorrhizal colonisation and frequency of root rot on Eastern White Pine seedlings at a southern Canadian nursery revealed a negative correlation between *T. terrestris* and root rot. This suggested that the association of this ectomycorrhizal fungus with *P. strobus* roots might have some antipathogenic effects (Ursic *et al.*, 1997). Additionally, removal of the basidiome of the ectomycorrhizal fungus *Laccaria bicolor* associated with container-grown Eastern White Pine seedlings induces a very rapid decrease in both net photosynthesis and stomatal conductance of the host plant (Lamhamedi *et al.*, 1994).

## 7. Domestication

Eastern White Pine has been an attractive species for planting within its range, with up to 40 million seedlings shipped yearly for fibre production and Christmas trees (Eckert and Kuser, 1988). The species has also been used for shelter-belts and urban plantings, and has been used on a small scale in some European countries. Despite its very high timber value, management difficulties with control of white pine weevil and blister rust in planted stands have discouraged its use. Eastern White Pine is thus a rather minor reforestation species, particularly in the northern parts of its range in Canada, where annual nursery shipments in Ontario, Quebec and the Maritimes are now well below 5 million. Nevertheless, the potential value of white pine planting and breeding is well recognised, and tree improvement programs for the species are maintained at some level throughout most of its range.

### A. Deployment of reforestation materials

White pine has a long history as a species for reforestation, and nursery production techniques are well-established. In the early years, most planting stock were produced as bareroot seedlings (Coons, 1978), with 2+0 shipped from southern nurseries and 3+0 in the north, although 2+2 transplants have demonstrated superior performance in the field (Mullin and Howard, 1973; Mullin and Christl, 1982). Following developments in nursery technology, Eastern White Pine is now commonly produced from seed in containerised systems, in soil-less growing media. A variety of containers are used and stock is raised in both heated and unheated greenhouse structures. Cultural techniques have become highly sophisticated, ensuring that high-quality planting stock can be produced reliably and efficiently (Landis *et al.*, 1989, 1990a, b, 1992).

Eastern White Pine planting stock can also be produced by means of vegetative propagation. Much of the research in this area has been motivated by possible clonal deployment of individual genotypes with putative resistance to white pine weevil and blister rust. While older trees are often difficult to propagate using long-shoot cuttings, those from 2- to 3-year-old seedlings have long been known to root easily (Deuber, 1942; Patton and Riker, 1958b; Zsuffa, 1973; Kiang *et al.*, 1974; Kiang and Garrett, 1975; Struve and Blazich, 1982). Propagation is also possible using fascicular shoots (Struve and Blazich, 1980, 1984). Growth and performance of rooted cuttings are comparable to planting stock raised from seed (Struve *et al.*, 1984; Struve and McKeand, 1990).

Clonal propagation of Eastern White Pine can also be achieved through micropropagation of juvenile explant cultures derived from cotyledons, epicotyls and hypocotyls (Kaul, 1987, 1990; Webb *et al.*, 1988). Techniques for the initiation of somatic embryos are also available, although whole plants have not yet been successfully recovered from these cultures (Becwar *et al.*, 1988; Finer *et al.*, 1989).

Some successful trials have demonstrated the potential of direct seeding as a regeneration technique for Eastern White Pine (Graber and Thompson, 1969; Horton and Wang, 1969; Graber, 1988), but stocking is often irregular (Torbet *et al.*, 1995). Operational use has generally been regarded as a failure and is not recommended (Waldron, 1974). Feeding losses to small mammals can be over 80%, unless the seeds are covered with soil at time of sowing (Graber, 1969).

## B. Provenance transfer

Local seed sources are often not the preferred provenance for planting, and northerly transfers are often beneficial, except in the extreme. Sources from the southern Appalachians perform well in all but the most northerly locations, with high volume production and reduced branchiness (Sluder, 1963; Funk, 1971, 1979; Sluder and Dorman, 1971; Funk *et al.*, 1975; Wendel and Cech, 1976; Wright *et al.*, 1976, 1979; Kriebel, 1978; Williams and Funk, 1978; Funk and Jokela, 1979). However, faster-growing southern sources are not sufficiently hardy to thrive in the harsher continental climates above 41°N (Fowler and Heimburger, 1969b; King and Nienstaedt, 1969; Jeffers, 1977). The use of seed zone controls to limit the transfers within regions of adaptation have been recommended for the northern part of the species range in Québec (Li *et al.*, 1997).

Tests in Australia indicated that the best provenances are from the southern part of the natural range, although none are as productive as *Pinus radiata* (Matheson, 1977; Wright *et al.*, 1979). In the Lower Saxony region of Germany, Appalachian Mountain sources below 39°N perform consistently well, while those from north of 45° perform poorly (Stephan, 1974; Genys *et al.*, 1978).

In most of Europe, North American pines are considered to be fast growing tree species. In Romania, *Pinus strobus* is the second most productive species after Douglas fir, and has the least variation in annual radial increment and the lowest wood specific gravity of any commercial species. It is recommended on rotations of 40-60 years for pulpwood and 60-80 years for saw timber (Radu and Radu, 1972). In contrast, despite the extensive introduction and promising performance of *P. strobus* in Bohemia and Moravia, its wood has been grossly underrated by the woodworking industry, largely as a result of premature felling (Vytiskova, 1970). Of the 20 exotic Pines (9 from North America) growing in the central chernozem region of south central Russia, *P. strobus* has the fastest growth rate. However, exotic pines grown in Russia are significantly inferior in growth rate and yield to the local *P. sylvestris* (Lutkin *et al.*, 1974). In the Lower Saxony region of the former German Federal Republic, *P. strobus* is not recommended for pure stands, partly because of the poor price paid for its timber and the unsaleability of thinnings; however, because of its fast growth, pleasing appearance, windfirmness, hardiness and general adaptability, it is strongly recommended for mixtures and particularly for the rehabilitation of recreation forests (Schumacher, 1974). As well, in provenance tests established in 1960 in Lower Saxony, growth of the best provenances of *P. strobus* was comparable or superior to that of local *P. sylvestris*, contrary to the situation in Russia (Stephan, 1981). Additionally, at two sites in Lower Saxony, differences between a rangewide sample of North American provenances were observed in height growth and mortality and attack by *Chronartium ribicola* (Stephan, 1974). *P. strobus* is recommended for wet or periodically waterlogged sites in the lowlands and hills of medium to low fertility in the former German Democratic Republic, especially those of extreme frost hazard (Thomasius and Hartig, 1979).

## 8. Summary

Eastern White Pine is one of the most important tree species in eastern North America. It has the largest range of any North American species in subsection *Strobus*, the "white pines", and is the only representative on the eastern side of the continent. It is an outcrossing, wind-pollinated species that can transfer genes rapidly to neighbouring populations and to other related species. Eastern White Pine is regarded as intermediate in its tolerance to shade, and natural regeneration is favoured by silvicultural systems that encourage partial shade during establishment and initial development.

Eastern White Pine exhibits clinal variation patterns, generally correlated with latitude, although local seed sources are often not the best performers. Heritability estimates are moderately high at young ages and, while typically decreasing at older ages, are sufficient to predict considerable gains from recurrent

selection. Significant genotype-environment interactions have been reported, but family ranks are generally stable across environments.

Best production is on medium-textured, well-drained soils, in cool, humid areas. White pine can occur as pure stands, or in mixture with several other conifer and hardwood associates, depending on site conditions and history of disturbance. It is a long-lived, successional species, but can be an aggressive pioneer on old fields. The white pine weevil and white pine blister are serious pests and are the major challenge for management of both natural and planted populations.

Eastern White Pine is well-suited to artificial regeneration and it has a long history as a planted species throughout its natural range, both in forestry and urban applications. Tree breeding efforts have been targeted primarily at selection and interspecific hybridisation, in an attempt to produce varieties with resistance to the weevil and blister rust. Management difficulties have limited planting of Eastern White Pine, particularly in the north of its range, although seed orchards are maintained in all regions. Meanwhile, a long history of economic exploitation has resulted in fragmentation and reduction of population sizes in some areas, making genetic conservation of this species a growing concern.

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## SECTION 2 EUROPEAN WHITE BIRCH (*BETULA PENDULA* ROTH)

### 1. General Description and Use in Forestry

#### A. Taxonomy

##### *Taxonomy*

European white birch, or silver birch (*Betula pendula* Roth), belongs to the genus *Betula*. There are some 40 *Betula* species, which are distributed throughout the northern temperate region. According to the systematics created by Regel, 1876 (in Natho 1957), the *Betula* genus is divided into two main sections, *Eubetula* and *Betulaster* (Table 3.3). The *Betulaster* section includes just a few birch species in Japan and China, in the subsection *Acuminatae*. The *Eubetula* section is further divided into three subsections, *Costatae* (yellow birches), *Albae* (white birches) and *Nanae* (dwarf birches). *Betula pendula* belongs to the *Albae* subsection, as does the other European treelike birch, *B. pubescens*.

In 1753 Carl von Linné defined the European arboriform birches as one species, *B. alba*. At the end of the century the German botanist, A. W. Roth, characterised the European white birch as its own species, *B. pendula* and this is the name used today, instead of *B. verrucosa* suggested by F. Ehrhardt (Raulo 1981). An Asian birch species, *B. japonica* is very close to *B. pendula*, since the hybrids of the two species produce fertile off-spring and the species are morphologically very similar (Johnsson 1945).

There are many variations and forms of *B. pendula* (Fontaine 1970). Most of the special forms of *B. pendula* var. *pendula* are grown as ornamental trees in parks and homesteads. An important variation economically is var. *carelica*, curly-birch. Its wood is strong and decorative and is used for making wooden ornaments. The wood is sold according to weight, and its price makes it more valuable than ordinary birch (Ryynänen and Ryynänen 1986).

##### *Chemotaxonomy*

The chemicals in birch stems are useful in recognition of different birch species. Julkunen-Tiitto *et al.* (1996) compared concentrations of 12 secondary metabolites in birch seedlings and saplings. Of phenolic compounds, dehydrosalidroside was found specifically in *B. pendula*. Platyphylloside was also a typical component in *B. pendula* and was found also in *B. papyrifera* but not in *B. pubescens*. Triterpenes are found in the resin glands on the surface of young birch stems. *B. pendula* contained mainly papyriferic acid (as did *B. papyrifera* and *B. platyphylla*) accompanied with deacetyl papyriferic acid. The secondary product composition in *B. pendula* differs markedly from that of, for example, the morphologically similar *B. pubescens* by the presence of platyphylloside and terpenoids. On the other hand, *B. platyphylla* (Japanese white birch) and *B. resinifera* show a moderately close phenolic and terpenoid relationship with *B. pendula*. Besides the chemical composition of the stem, foliar chemistry is also used for recognition of birch species. The end product of the ellagitannin pathway, 2,3-(S)-HHDP-glucose accumulates in the leaves of *B. pubescens* and *B. nana*, whereas it is present only in trace amounts in the leaves of *B. pendula* (Salminen *et al.* 2002). These results show that, together with exomorphic and cytological features, chemo-

taxonomical comparison of secondary components can be used to distinguish between species or varieties (Julkunen-Tiitto *et al.* 1996).

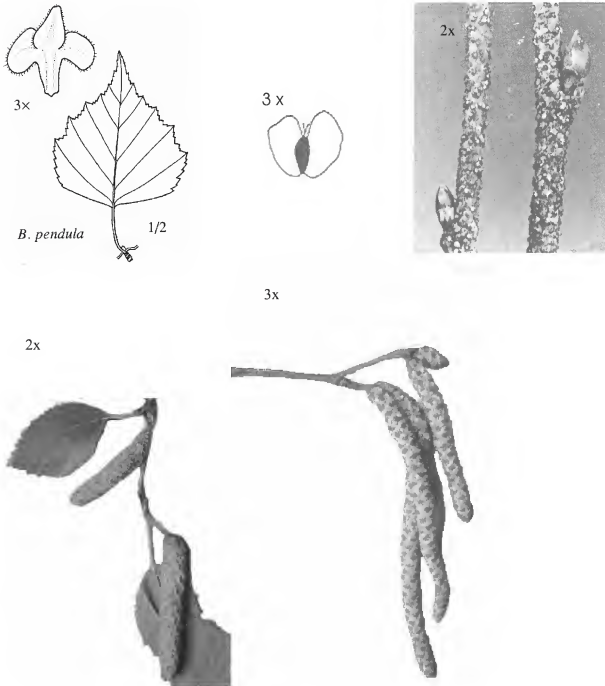
**Table 3.3 The taxonomy and distribution of the 44 species in the genus *Betula* (Fontaine 1970; Raulo, 1981). The taxonomy of the genus *Betula* is under dispute (Flora Europaea, 1993)**

Genus	<i>Betula</i>			
Section	<i>Eubetula</i>			<i>Betulaster</i>
Subsection	<i>Costatae</i>	<i>Albae</i>	<i>Nanae</i>	<i>Acuminatae</i>
Species	<i>B. costata</i> Trautv. <sup>1</sup> <i>B. globispica</i> Shiras <sup>1</sup> <i>B. medwediewii</i> Regel <sup>1</sup> <i>B. utilis</i> D. Don <sup>1</sup>  <i>B. ermanii</i> Cham. <sup>1</sup> <i>B. chinensis</i> Maxim. <sup>1</sup> <i>B. schmidtii</i> Regel <sup>1</sup> <i>B. potanini</i> Shirai <sup>1</sup>  <i>B. albo-sinensis</i> Burk <sup>1</sup>  <i>B. alleghaniensis</i> Britt. <sup>2</sup> <i>B. lenta</i> L. <sup>2</sup> <i>B. nigra</i> L. <sup>2</sup>  <i>B. grossa</i> Sieb. & Zucc. <sup>1</sup> <i>B. jacquemonti</i> Spach <sup>1</sup> <i>B. coryifolia</i> Regel & Maxim. <sup>1</sup>	<i>B. japonica</i> Sieb. <sup>1</sup> <i>B. mandshurica</i> Nakai <sup>1</sup> <i>B. davurica</i> Pall. <sup>1</sup> <i>B. papyrifera</i> Marsh. <sup>2</sup>  <i>B. populifolia</i> Marsh. <sup>2</sup> <i>B. pendula</i> Roth <sup>1,3</sup> <i>B. pubescens</i> Ehrh. <sup>1,3</sup> <i>B. platyphylla</i> Sukatchev <sup>1</sup> <i>B. caerulea-grandis</i> Blanchard <sup>2</sup> <i>B. occidentalis</i> Hook. <sup>2</sup> <i>B. minor</i> Fern. <sup>2</sup> <i>B. concinna</i> Gunnarsson <sup>3</sup> <i>B. coriacea</i> Gunnarsson <sup>3</sup> <i>B. microphylla</i> Bge <sup>1</sup> <i>B. turkestanica</i> Litvinoff <sup>1</sup>	<i>B. nana</i> L. <sup>1,3</sup> <i>B. fruticosa</i> Pall. <sup>1,3</sup> <i>B. exilis</i> Sukacz. <sup>1,2</sup> <i>B. middendorffii</i> Trautv. & Mey <sup>1</sup> <i>B. pumila</i> L. <sup>2</sup> <i>B. michauxii</i> Spach <sup>2</sup> <i>B. glandulosa</i> Michx. <sup>2</sup> <i>B. humilis</i> Schrenk <sup>1,3</sup>  <i>B. glandulifera</i> Butler <sup>2</sup>  <i>B. hallii</i> Howell <sup>2</sup> <i>B. uber</i> (Ashe) Fern. <sup>2</sup>	<i>B. maximowicziana</i> Regel <sup>1</sup> <i>B. alnoides</i> Buch.-Ham. <sup>1</sup> <i>B. luminifera</i> Winkl. <sup>1</sup>
Distribution	Asia <sup>1</sup> North America <sup>2</sup>	Asia <sup>1</sup> North America <sup>2</sup> Europe <sup>3</sup>	Asia <sup>1</sup> North America <sup>2</sup> Europe <sup>3</sup>	Asia <sup>1</sup>

## B. General description

*Betula pendula* grows up to 30 m high, with usually one stem. It can become up to 100 years old, under favourable conditions even 150 years. The bark is smooth and silvery white and exfoliates in long, thin strands. The basal parts of the trunks of old trees are black and vertically fissured; in young trees (up to 10 years old) the bark is light brown. Older branches are often pendulous, whereas young branches are spreading or ascending. Twigs are glabrous, with peltate resin glands on the younger twigs. Buds are acute, not sticky. Leaves are 2.5 – 5 cm long, ovate-deltate, apex acuminate, base truncate or broadly cuneate. Leaf margins are double serrated with the primary teeth very prominent and curved towards the leaf apex; petioles are 10 – 18 mm long. The scales are short, with a broad cuneate base, broad lateral lobes spreading and curving towards the base, and a deltoid, obtuse middle lobe. The achene is glabrous, 1.5 – 2.4 mm long, 0.8 – 1.3 mm wide; the width of the fruit is 3 – 5 mm, the upper edge of the wings surpassing the stigmas by 0.5 – 1.5 mm (Atkinson 1992; Figure 3.2.).

Figure 3.2 The leaf, scale, seed, stem, buds and female and male catkins of *Betula pendula* (Raulo 1981, Hämet-Ahti *et al.* 1992).



### C. Use of European white birch

#### *Firewood*

Birch has been an important source of energy, and earlier it was mostly used as firewood. The effective thermal value of birch is about 20 MJ/kg when absolutely dry, and about 15 MJ/kg with 30 % humidity. The high content of volatile oils makes the burning properties good (Salmi 1987).

#### *Plywood production*

In plywood production, the value of a birch log used for planks or veneer is influenced by its diameter, stem taper, and number and size of knots and branches. Logs of required dimensions can usually be produced with proper planting densities and thinning treatments.

#### *Pulpwood*

Birch fibres are relatively long and thin-walled and have a high content of hemicelluloses. As a result, birch pulp has good strength properties, low light-scattering ability and high density. The main product is bleached sulphate pulp. Coated and uncoated wood-free fine papers, which contain 50 to 90 % of bleached birch sulphate pulp, are especially important (Tammisola *et al.* 1995).

#### *Curly-birch, Carelian birch*

Curly-birch (*B. pendula* var. *carelica*) is a special form of *B. pendula*, and it occurs throughout Northern Europe and parts of Central and Eastern Europe. The wood is strong and decorative and is used in wooden ornaments and furniture. The genetic background of the curly-grained trait is not known. Curly-birch is self-sterile, and in controlled crossings between two individuals a maximum of 75 % of the progeny can be curly-birch. It has been suspected that the trait is homozygotically lethal, or that it is a genetical disease caused by a micro-organism or a virus (Ryynänen and Ryynänen 1986). The establishment of a plantation is an uncertain process, because it takes 10 years to see whether an individual will become a curly-birch. Normal birches have to be removed in order to prevent the curly-birches becoming suppressed. The propagation of curly-birches from cuttings has been attempted but the results have not been promising. Micro-propagation techniques have been developed for propagating curly-birches with the optimum stem form (Ryynänen and Ryynänen 1986).

## 2. Forestry Practices

### A. Reproductive methods

#### *Flower induction*

In order to speed up the induction of flowering, and consequently, breeding, the Foundation for Forest Breeding in Finland built the first flower induction hall in 1976. It was equipped to provide good growing conditions for breeding material all year round. In the plastic greenhouses continuous illumination (24h day length) was kept at 10,000 – 20,000 lux by mercury and high pressure sodium lamps. Temperature was controlled by thermostats and fans. CO<sub>2</sub> concentration was increased by burning propane gas and relative humidity was kept above 60 %. Required nutrients were provided automatically via continuous drip irrigation. The best results were obtained from birch: the daily growth was 7 cm per day, male flower buds appeared in 77 days and the first seed crop was collected 8 months after sowing (Holopainen and Pirttilä 1978).

## B. Vegetative propagation

Birch can be propagated vegetatively, *i.e.* cloned, by grafting, rooting or by means of tissue culture. Production of grafts is quite expensive and grafts are used mainly for clonal collections and seed orchards (Ryynänen 1987). Also, rooting of birch cuttings is not much used in birch propagation as the percentage of rooted cuttings is low (10 – 50 %) for *B. pendula*, whereas Kling *et al.* (1985) found rooting rates of 80 % for a closely-related *B. platyphylla*.

### *Micro-propagation*

Much research on tissue culture of birches has been done and effective micro-propagation techniques have been developed. Leaf callus from young seedlings can be used (Simola 1985) but with mature trees, vegetative buds are used as tissue material (Ryynänen and Ryynänen 1986). Shoots and roots can be specifically induced on different aseptic culture media, after which the plantlets can be potted on peat and moved into the greenhouse. The development of micro-propagation methods made an effective propagation of selected adult trees possible. Micro-propagated plants have proved viable in test plantings under field conditions (Meier-Dinkel 1992; Jones *et al.* 1996). A review on micro-propagation of juvenile and mature birches including results on field trials and cold storage was compiled by Meier-Dinkel (1992). Some clonally propagated birch plants have been used for practical forest cultivation in Finland (Viherä-Aarnio 1994a). Large-scale production of micro-propagated *B. pendula* seedlings was terminated in 1994, as it was considered unprofitable (Viherä-Aarnio and Velling 2001). Today, micro-propagation is used in commercial scale for curly-birch cloning. Micro-propagated plants can also be used instead of grafts in greenhouse seed orchards, when specific genotypes are required (Viherä-Aarnio and Ryynänen 1995).

Viherä-Aarnio (1994a) studied the field performance of micro-propagated birch plants in a small-scale field test with three clones and three seed-born lots. The best two lots of the experiment as regards the height and diameter growth at the age of six years were clones, but the weakest lot was also a clone. This indicates that the selection for clonal propagation should be done carefully. It is also important to test the clones in field trials before wide scale propagation. In fact, further studies revealed that micro-propagated clones do not differ from seed-born seedlings in terms of growth and resistance against pests and herbivores (Viherä-Aarnio and Velling 2001).

## C. Reproductive materials used

### *Seed collection stands*

Seed collection stands are natural birch stands of outstanding growth and quality. The best individuals in these stands have been selected as plus trees. A part of the seed used in nurseries is collected from seed collection stands (Viherä-Aarnio and Ryynänen 1994).

### *Seed orchards*

As birch responds positively to intensive cultivation in a greenhouse, today most birch seed used for seedling production is obtained from polythene greenhouse seed orchards, a method which was developed by the Foundation for Forest Tree Breeding in Finland in 1972 (Huhtinen & Yahyaogly 1974). Most seed orchards are multi-clonal orchards with some 30 to 50 clones. Bi-clonal orchards have also been established in order to produce desired full-sib families (Viherä-Aarnio and Ryynänen 1994). Besides greenhouse orchards, open seed orchards in the field are used for seed production.

Seed production in greenhouse seed orchards begins three years after planting and continues for 5 to 7 years, until the trees grow too big to be kept inside. Flowering and seed ripening take place 1 to 2 weeks earlier in the greenhouse than in natural conditions. As a result, growing birches in greenhouses also

prevents unwanted background pollination. A seed orchard can be established with grafts of tested plus trees, seedlings selected from the best progenies or micro-propagated clones (Viherä-Aarnio and Ryyänänen 1995).

### ***Cultivation***

In the Nordic countries and Europe, Finland has the longest experience in birch cultivation. The main emphasis in birch planting has been the afforestation of former agricultural land (Ferm *et al.* 1994). When a field is abandoned, it is first colonised by annual species, which then give way to perennial herbs and grasses. As field vegetation competes with tree seedlings for water and nutrients, success with field afforestation requires effective weed control during the establishment year. Soil preparation also affects the survival of trees. In 20-year-old experiments the best growth was gained when bare-rooted, large transplants of silver birch were planted on a ploughed and tilled field and weeds were properly controlled (Torpo 1991). Weed control improves the nutrient status of tree seedlings and increases growth. Birch seedlings with efficient weed control were 40 – 50 cm taller after two years than control seedlings, and they had bigger leaves (Ferm *et al.* 1994). Vegetation control reduces the risk of cicada (*Cicadella viridis*) wounding and consequently infection by pathogenic fungi, and is also effective against vole damage (Ferm *et al.* 1994).

### **D. Breeding**

In the breeding programme for birch, outstanding stands for seed collection were selected and the best individual trees in these stands were selected as plus trees. The most important selection criteria were fast growth and good stem quality. After selection of plus trees, controlled crossings have been carried out, and progeny tests with both full-sib families from controlled crosses and half-sib families from open pollinated lots have been established (Koski 1991). Plus tree selection is still being continued in order to improve the geographical coverage of the breeding material (Viherä-Aarnio 1994b).

Planted birch stands of improved material can reach the size of final cutting by as early as 40 years and produce over 400 m<sup>3</sup>/ha (Viherä-Aarnio 1994b). Examples of realised genetic gain cannot yet be shown, but a potential gain of 20 to 30 % in volume growth has been suggested (Koski 1991) and in first generation seed orchards, the growth rate of the best selected family is 89 % higher than the control (Wang 1996).

### ***Provenance transfers***

Raulo and Koski (1977) reported on the first geographical transfers of silver birch. According to their study, seed transfers of 200 km northwards or southwards in central and southern Finland had no effect on the mortality or growth rate of the progenies; on the other hand they did not recommend long-distance transfers because of the risk of lower survival.

### **E. Conservation of genetic resources**

#### ***Genetic diversity of forest trees***

The cultivation of forest trees can be thought to endanger the genetic diversity in general. Maintaining genetic diversity in forest tree populations also means maintaining adaptability to changing environments. In naturally generated areas there is no concern about the loss of genetic diversity but large monocultures of a single clone would reduce genetic variability. However, forestry based on monoclonal blocks is not common, but cultivation of single clones is mostly applied to small plantations of special forms or variations, *e.g.* curly-birch (Viherä-Aarnio and Velling 2001). Moreover, real monocultures are impossible to maintain, as other trees can not be prevented from growing in the plantations.

Most forest trees are cross-fertilising and pollinated by wind and consequently there is crossing both between individuals in a stand and between stands. Even if small stands were to be cultivated with only one clone, effective wind-pollination will secure the mixing of genes between populations. Also, real monocultures are impossible to maintain, as other trees can not be prevented from growing in the plantations. Cultivation of single clones will probably mostly apply to small plantations of special forms or variations, *e.g.* curly-birch. The danger from planting large areas with a single clone arises when the adaptability of the clonal genotype is exceeded by adverse conditions. To avoid multiplication of poorly adapted or extremely susceptible genotypes, trees for micro-propagation are selected from old stands. Another option is the use of clonal mixtures which are buffering environmental risks (Kleinschmit 1998).

It has been estimated that a population of 500 trees is big enough to contain all possible alleles of different genes (Koski 1995), and this is the amount usually used in breeding populations. The seed from seed orchards is usually a result of cross-fertilisation of tens of plus tree clones from different localities, and it has been shown in studies with genetic markers that the genetic diversity is as large as that of natural seed collection stands (Koski 1995).

### *Gene reserve forests*

Ongoing breeding activities may cause losses of genetic diversity, especially if population size is reduced from one generation to the next. Random allele loss is problematic for advanced-generation elite populations, and this has raised concerns about the maintenance of genetic diversity in forest-tree breeding programs.

Gene reserve forests have been established in order to conserve representative samples of the natural gene pool of forest trees. They have to be big enough (preferably tens of hectares) for the pollination to occur inside the forest and for the reserve to contain most of the local genetic diversity. Management and harvesting are allowed, but the forests are regenerated naturally or by sowing with seed from inside the forest or by planting seedlings grown from local seed. Gene reserve forests should consist of a network of forests in the area of the natural distribution of the species, in order to contain the diversity between provenances and localities. In Finland the gene reserve forests are situated in northern Finland and are part of a network of gene reserve forests covering all Europe (Parviainen *et al.* 2000).

### *Advanced breeding strategies*

Two advanced models have been suggested in order to prevent random allele loss in breeding programs: HOPE (Hierarchical Open-Ended Breeding System) and MPBS (Multiple-Population Breeding Strategy) (Eriksson *et al.* 1993).

The HOPE system is composed of a hierarchy of breeding populations with successively higher performance levels. A large base population is maintained and is open to new material. Higher level populations are more stringently selected and from these, selections are made for commercial production. Under MPBS, a breeding population is divided into independent subpopulations which represent different sources or selection criteria. There is differentiation among subpopulations both in their source of germplasm and in their traits and environmental adaptabilities. There can be random allele loss in some subpopulations but, on average, gene frequencies remain fairly constant (Eriksson *et al.* 1993).

Of these, the MPBS seems to be a better choice for birch because it combines the highest possible genetic gains and the highest possible genetic diversity. It also gives the breeder more options for changing breeding goals with changing environment and markets (Eriksson *et al.* 1993).

### 3. Centres of Origin / Diversity

#### A. Natural distribution and origin

##### *Natural distribution*

*B. pendula* is distributed throughout Eurasia (Figure 3.3.). It extends in Europe into the mountainous regions of Spain, Greece and Italy in the south, throughout Asia all the way to the Sea of Okhotsk and the Sea of Japan in the east and to Caucasus, Crimea and the mountains of Central Asia in the south. In the north it extends to about 65°N (Hämet-Ahti 1963; Atkinson 1992).

**Figure 3.3** The natural distribution of *Betula pendula*



Source : Hämet-Ahti *et al.*, 1992

The northern limit of the range of silver birch appears to be determined by protection from cold north-easterly winds. The southern limits approximate to the line of an average of 10 mm July rainfall (Atkinson 1992). There are different views of the eastern limit of the range, 60° by Jalas & Suominen (1976) and 103° by Hämet-Ahti *et al.* (1992).

##### *Total distribution*

In North America *B. pendula* is used as an ornamental and is a naturally spreading escape from cultivation for example in Ontario, Canada (Catling and Spicer 1988). The corresponding species of *B. pendula* in North America is *B. populifolia* (grey birch) which is used in re-vegetation of mine spoils, and is economically important as a source of fuel, sugar and fibre for corrugating pulp (Catling and Spicer 1988).

##### *Migration to Fennoscandia*

Birch migrated to Fennoscandia in the late glacial with pine, spruce and alder. The last remnants of the Scandinavian ice cap may have disappeared at around 8,000 years ago and the early postglacial (pre-boreal) forests in Fennoscandia were dominated by birch. In the boreal (6,800 – 5,000 BC), the climatic conditions were drier and cooler, and pine replaced birch especially in the north. At around 5,000 BC, the climate became warmer again, and birch and alder spread to new areas. At around 1,000 BC, as the climate became cooler again, spruce replaced birch and pine in many areas (Hämet-Ahti 1963).

#### 4. Reproductive Biology

##### A. Sexual reproduction

Birches are monoecious (male and female flowers on the same tree) and diclinous (flowers unisexual). Male inflorescences are 1–2 cm long, 4 mm wide while over-wintering; 2–6 cm long, 6 mm wide at anthesis. There are 2–4 male catkins together at the ends of small shoots. Female inflorescences are erect, 1.5–3.5 cm long, 7 mm wide (Atkinson 1992). They are pale green when immature, turning brown in the late autumn.

##### Flowering

Flowering usually starts when trees are between 5 and 10 years old but some single trees can be induced to flower within one year after germination (Elo *et al.* 2001). The female flowers emerge on the short-shoots at the same time as the buds open. The female flowers usually develop one day before male flowers on the same tree shed pollen. Flowers remain receptive for 4 days and after the sixth day are blackened and dead (Sarvas 1952). *B. pubescens* flowers about a week later than *B. pendula*.

Ripening of the flowers is closely related to the temperature conditions of the spring. The arrival of the first heat wave of daily temperatures above +10°C usually triggers flowering. Most pollen release occurs within 2 or 3 days of anthesis. Pollen shed is highest in the late afternoon and near zero from about midnight to early morning (Sarvas 1952). *B. pendula* pollen can remain viable for at least 20 days at room temperature in the dark. If the pollen is exposed to sunlight for 8 hours, however, ultraviolet rays and other factors reduce viability (Perala and Alm 1990).

Flowering is polygenically inherited and female and male flowers are governed by different sets of genes. Weather conditions have a strong influence on female flowering whereas they do not play a role in male flowering (Eriksson and Jonsson 1986).

Three cDNAs of *B. pendula* representing ADS-box genes BpMADS3, BpMADS4 and BpMADS5 are active during the development of both female and male inflorescence and the respective genes are involved in the determination of the identity of the inflorescence or flower meristem. BpMADS3 shows highest expression at late developmental stages. BpMADS4, besides regulating inflorescence, is expressed in roots and stem. Expression of BpMADS5 is inflorescence specific and continues during seed development. Ectopic expression of BpMADS3, BpMADS4 or BpMADS5 with CaMV 35S promotor in tobacco results in extremely early flowering (Elo *et al.* 2001).

##### B. Pollination

##### Male meiosis and pollen development

The male catkins develop in the autumn but do not release pollen until spring the following year. The principal events during micro-sporogenesis are the initiation and growth of the male buds, generation and growth of the sporogenous tissue, pre-meiotic maturation of the PMCs (pollen mother cells), meiosis, the tetrad stage, androgenesis and anthesis. The species in *Betulaceae* reach the tetrad phase from late July to mid-August (Luomajoki 1986). In *Betulaceae* the heat sums seem to have less connection with the development stage of micro-sporogenesis than in conifers, as the northernmost stands of *Betula* species develop first and the southernmost last. The timing of meiosis in *Betula* is more dependent on day length, and a hypothetical critical day length of 15 hours can quite accurately predict the onset of the tetrad phase (Luomajoki 1986).

### **Pollination**

Pollination is anemophilous, *i.e.* birch is pollinated by wind. There is an intense maximum at the immediate start of flowering lasting 2 – 3 days during which 70 – 80 % of the total pollination takes place. Prolonged cold and wet conditions are likely to reduce the total amount of pollen (Sarvas 1948).

### **Pollen flow**

As an evidence of long-distance pollen transport, *Betula* pollen concentrations in Fennoscandia can be relatively high before the local flowering period. The pollen is transported by south-eastern air-masses from eastern central Europe and the Baltic countries, with travelling times for pollen grains in the range of 9 – 20 hours (Hjelmroos 1991).

In northern Europe, birch pollen allergy is one of the most common reason for spring-time human rhinoconjunctivitis and asthma. In Sweden, about 8 – 9 % of the population suffers from allergies related to birch pollen (Hjelmroos and van Hage-Hamsten 1993). The major birch pollen allergen Bet v I is highly homologous to pathogenesis-related plant proteins. Another well described allergen is profilin, an actin binding protein, which is predominantly expressed in the pollen of different plant species. The birch profilin is recognised by IgE antibodies in 10 % of individuals allergic to birch pollen and seems to be an intermediate or major allergen in individuals allergic to pollens of grasses and weeds (Valenta *et al.* 1991). Another birch pollen allergen, Bet v III, representing a class of Ca<sup>2+</sup> binding proteins, has also been characterised (Seiberler *et al.* 1994).

## **C. Mating system**

### **Cross-fertilisation**

*B. pendula* is an outcrossing species. There is cross-fertilisation both between trees standing close in the stand and among distant individuals in the same or nearby stands. Hagman (1971) studied incompatibility in *B. pendula* and *B. pubescens* and found partial incompatibility in both cross-pollinated and self-pollinated trees. When trees from the same population were cross-pollinated some combinations were incompatible. Incompatibility occurs in the style as retarded growth of the pollen tube and is probably based on a one-locus multiple allele system. Incompatibility reaction is also influenced by environmental conditions: compatibility is increased at low temperatures.

### **Self-fertilisation and inbreeding depression**

In the studies of Hagman (1971), self-incompatibility was observed after self-pollination. Self-incompatibility was not complete and the filled seed frequency was highest when the pollinations took place at low temperature.

Wang (1996) studied inbreeding depression in *B. pendula* and the possibility of using heterosis in breeding. He found that inbreeding depression in survival and in stem volume of progenies in three successive generations of selfing was significant. The mean survival rates were 88 %, 37 %, 28 % and 42 % for outcross controls, S1, S2 and S3 selfed progenies, respectively. Inbreeding depression for stem volume also increases with advancing generations of selfing. The mean stem volumes were 5.31, 3.15, 2.66 and 2.12 dm<sup>3</sup> for outcross controls, S1, S2 and S3 selfed progenies, respectively.

## D. Seed crop

### *Seed crops*

The percent of germinating seed is directly related to the amount of pollen shed: seed crops with the highest viability will be produced during years of abundant flowering (Sarvas 1952). Each strobile contains about 450 seeds. In the average year in ordinary regeneration areas, seed crop is some 340 filled seeds  $\text{m}^{-2}$ , with 2 300 seeds  $\text{m}^{-2}$  in a good seed year. In a good seed year in a pure birch stand the best annual result has been 53 200 seeds  $\text{m}^{-2}$ , i.e. 128 kg per hectare. A crop amounting to 20 % of the best seed crop occurs only every third year, and intervening crops may be only 5 – 10 % of a good year (Sarvas 1948). Cold springs can lead to massive loss of male catkins. Birch produces empty fruits in the absence of pollination.

Dispersal range of birch seed is limited by the small seed size. The fall rate for silver birch seed is  $0.52 \text{ m s}^{-1}$  (Sarvas 1948). Most seeds fall within 40 – 50 m of the source, with a maximum amount of seeds at 10 – 12 m from the tree. The amount of seeds is still 5 % of the maximum at 100 m from the source, but after 70 m most seeds are empty. In *B. pendula* seeds are also dispersed throughout the autumn and winter, so there is probably also secondary dispersal of seed over the surface of snow by wind. With *B. lenta*, seeds were distributed over an area 3.3 times greater than by initial seed fall, and further dispersal by melting water in the spring could be some 50 km (Matlack 1989).

## E. Natural regeneration

### *Germination and establishment in the field*

A non-fluorescent water-soluble substance in the seed coat has germination-inhibiting properties. This inhibitor apparently increases the oxygen and light requirement of the embryo. Un-chilled, intact seeds require light for germination, but breaking the seed coat allows rapid entry of oxygen and hastens germination in darkness (Perala and Alm 1990). A long-day photoperiod or illumination with red light induces germination, whereas blue or far-red light inhibits germination. Stratification (chilling) or temperatures above  $20^{\circ}\text{C}$  override the photoperiod requirement. Fluctuating temperatures within the range of  $20 - 30^{\circ}\text{C}$  are more favourable for germination than steady temperatures (Perala and Alm 1990). Stratification also eliminates the effect of temperature on germination rate (Atkinson 1992). In natural conditions in the northern parts of the range of birch, the cold requirement is usually met.

In the autumn, after seed shed, short days and low temperatures inhibit germination and help seed survival over the winter. In the spring, after a long enough chilling period, dependence of germination on day length disappears and seeds germinate when temperature rises and there is enough moisture in the ground (Nygren 1987). Birch seeds may also germinate soon after seed deposition in the summer, if there is enough moisture in the ground. As the individual seeds are small and contain small amounts of reserve food, the radicle of the birch seedling remains short with the result that the plant frequently withers away and dies and seedlings only grow up on spots which contain enough moisture.

The percentage of germination of *B. pendula* seed in southern Finland ( $60^{\circ}\text{N}$ ) is about 61 % and it decreases towards the North, being about 53 % in central Finland ( $62^{\circ}\text{N}$ ). The opposite is true for *B. pubescens* with germination of 63 % and 70 %, respectively (Sarvas 1948).

In natural conditions the seeds are concentrated in the upper parts of the soil profile indicating a rapid turnover. Hill and Stevens (1981) showed that 80 % of the seeds were found in the litter layer and showed no long-term survival. In studies where *B. pendula* and *B. pubescens* seed were sown to two forest sites in northern Sweden, only 6 – 9 % of the seeds remained viable after one year. The depletion rate was slower in the two successive years (Granström and Fries 1985). This might be due to litter-fall and growth of mosses altering the environment, making the seeds less liable to germinate. When the seeds were buried

beneath the litter layer, the viability of the seeds did not fall consistently over a five-year period even though after three years the pericarps were more or less degraded exposing naked embryos (Granström 1987).

80 – 90 % of birch seedlings die within the first year, perhaps mainly as a result of drought. Establishment is best on bare mineral and humid soils and cushion Sphagnum. These surface types have the most favourable moisture conditions (Kinnaird 1974).

## F. Vegetative reproduction in the field

### *Sprouting*

Sprouting by basal buds occurs as a response to fire as well as other damage such as felling and grazing. The sprouting ability of *B. pubescens* is stronger than that of *B. pendula*. Sprouting ability differs seasonally, and is generally higher for dormant trees. Felled *B. pendula* produces the lowest frequency of stump sprouting in summer and the highest in fall and spring. The frequency of sprouting and shoot size (but not the number of shoots) depends on light intensity and temperature. No stumps were observed to sprout at temperature lower than 10°C (Perala and Alm 1990). Sprouting depends also on the soil type: sprouting is more frequent in moist forests and on peat land than on dry heath and woodlands. Sprouting also depends on the method of regeneration. It is most frequent on clear-cut areas and least frequent on group selection cutting areas, forest fire areas and burnt-over clear-cut areas because of damage to the basal buds (Sarvas 1948).

## 5. Crosses

### A. Inter-specific crosses

Natho (1957) found several natural crosses between *B. pendula* and other birch species in Germany, i.e. *B. pendula* x *B. pubescens*, *B. pendula* x *B. humilis* and *B. pendula* x *B. pubescens* x *B. humilis*. Moreover, the author showed that there is a continuum from *B. pendula* via *B. pubescens* to *B. humilis* in leaf shape. This and the triple hybrid indicate that there is gene flow among the three species.

Johnsson (1945) found large variation among experimental crosses of *B. pendula* and five other birch species. The percentage of fruits containing seed were 53.2 %, 8.8 %, 0.9 % and 0.4 % for *B. pendula* x *B. japonica*, *B. pendula* x *B. papyrifera*, *B. pendula* x *B. ermanii* and *B. pendula* x *B. maximowicziana*, respectively. The hybrid *B. pendula* x *B. pubescens* was infertile in this experiment.

Clausen (1970) carried out inter-specific crosses between 12 *Betula* species (Table 3.4.). He observed that high-ploidy female x low-ploidy male usually gave low seed germinability. Crosses within a subsection did not give more germinable seeds than crosses between species from different subsections. Crosses with *B. ermanii* as female gave more germinable seeds than any other cross. Each species was represented by 1 – 13 individuals, so definite conclusions can not be drawn from these results. However, it is the most comprehensive study of its kind to date.

**Table 3.4 A summary of the results on seed germination in different crosses between *Betula* species**

		COSTATAE				ALBAE				NANAE			
		len.	nig.	erm.	all.	pen.	pop.	pub.	pap.	gla.	hum.	nan.	pum.
<i>lenta</i>	2n		0	0	***	-	*	-	0	0	0		0
<i>nigra</i>	2n	*		***	*	**	*	**	*	*	**	**	**
<i>ermanii</i>	4n	***	*		0	***	*	***	***	0	***	0	***
<i>alleghaniensis</i>	6n	*	*	*		0	*	*	***	*			*
<i>pendula</i>	2n	*	*	***	*	***	*	***	***	**	*	*	*
<i>populifolia</i>	2n	*	*	*	*	*		*	*	*	*		*
<i>pubescens</i>	4n	0	*	*	*	*	*		*	*	***	0	*
<i>papyrifera</i>	4-6n	**	*	**	**	***	*	***		0			***
<i>glandulosa</i>	2n		0	-	0	0	0	***	-			-	-
<i>humilis</i>	2n	0	0	***	*	*	0	***	**	0			***
<i>nana</i>	2n	-	*	0		0	*	**	0	**			0
<i>pumila</i>	4n	0	0	*	0	*	*	*	*	0	*	0	

Germinability %: <10 low (\*), 10–30 moderate (\*\*), >30 high (\*\*\*), no seed set (-)

Hagman (1971) studied incompatibility in *B. pendula* and *B. pubescens*. Incompatibility between *B. pendula* and *B. pubescens* is more pronounced when *B. pendula* is the male parent. Moreover, the *B. pendula* pollen tubes do not seem to be able to penetrate the style of *B. pubescens*. Low temperature gave the highest frequency of successful hybridisations. The following natural inter-specific hybrids of *Betula* species have been found in Finland (Kurtto and Lahti 1987):

- *B. pendula* x *pubescens*; *B. nana* x *pendula*; *B. nana* x *pendula* x *pubescens*.
- *B. nana* x *pubescens*.

## B. Intra-specific crosses

Raulo and Koski (1977) studied long-distance crosses (distances between parent trees > 100 km) in Finland in order to find out if inter-provenance crosses would result in hybrid vigour, that is, in a heterosis effect. In these experiments crosses between Finnish provenances did not result in a heterosis effect.

Wang (1996) studied inbreeding depression and long-distance crosses in *B. pendula* and the possibility of using heterosis in breeding. He found heterosis both in hybrids of inbreeding progenies and in provenance hybrids. The growth performance of the long-distance crosses (central Finland x Estonia, northern Finland x Latvia, Germany x southern Finland, central Finland x Austria) significantly exceeded that of controls. The southern long-distance crosses also outperformed short-distance crosses (between southern and central Finland, southern Finland and Estonia, and southern and northern Finland), but did not significantly differ from the local inbred crosses. Hybrids of selfed progeny gave a significantly better growth performance than controls of either stand origin or conventional full-sib family origin: the mean stem volume of hybrids was 52 % higher, and the volume of the best hybrid family was 112 % higher than the mean of improved full-sib family controls. These results suggest that hybrids of inbred lines or provenances could be used in breeding of silver birch for superior yield.

## 5. Genetic Variability

### A. Genetic background

#### *Chromosome number*

The birches are characterised by their small chromosomes which are only a few micrometers long. A double-staining method is useful in counting the number of chromosomes in *Betula* and in other hardwood species with chromosomes of small size and large number (Hömmö and Särkilahti 1986).

*Betula pendula* is a diploid with 28 chromosomes; *B. pubescens* is a tetraploid with 56 chromosomes. Trees morphologically resembling tetraploid but with intermediate chromosome number ( $2n=42$ ) are sometimes found. Because the chromosomes tend to lie in groups of seven and because of small numbers of quadrivalents in meiosis in the 28 and 56 chromosome plants, the original basic chromosome number is thought to be seven rather than fourteen (Eriksson and Jonsson 1986).

Natural polyploidy is very frequent in the genus *Betula* and the number of ploidy levels differ between the four subsections of the genus. *Costatae* has diploid ( $2X=28$ ), tetraploid ( $4X=56$ ) and hexaploid ( $6X=84$ ) species and in addition to these, three ploidy levels. *Albae* has also a pentaploid ( $5X=70$ ) species, *Nanae* has both diploid and tetraploid species and *Acuminatae* only diploid ones. *Betula* is a young species from the evolutionary point of view which explains the polyploid nature and the occurrence of various ploidy levels (Särkilahti and Valanne 1990).

The possibility of using induced polyploidy to speed up the natural evolution and breeding of *Betula* arose in the 1960s (Särkilahti and Valanne 1990). The polyploidisation experiments were performed by treating seeds with colchicine during germination. Of the 687 polyploid trees produced by these experiments in Turku, Finland, 287 were still alive in 1990. Moreover, a series with ploidy levels ranging from diploid to dodecaploid ( $2X - 12 X$ ) consisting of both natural and induced polyploids is available (Särkilahti and Valanne 1990).

Identification of polyploid trees of *B. pendula* is possible visually on the basis of leaf morphology, with polyploid trees having larger leaf blades, thicker leaf petioles and a rougher network structure on the abaxial leaf epidermis than normal trees. The mortality of colchicine polyploid trees is typically high in every growth phase, growth rate is slow and growth habit more or less abnormal. Thus, colchicine-induced autopolyploid trees are not of great value as such but they can be used for studying the effect of the ploidy level on growth, breeding, adaptability and evolution of *Betula*. In natural conditions polyploids are said to possess greater ecological and genetic amplitude and, therefore, exhibit greater variability than related diploids (Särkilahti 1990). Ploidy manipulation seems to produce mainly sterile trees but micro-propagation can be used for multiplication of polyploid material.

### B. Variability within and between populations

Long-lived species such as forest trees are subject to conditions varying greatly from year to year. Thus, they have a large within population variation so that there are always genotypes well adapted to the varying conditions at regeneration. For example, in studies of five year heights of *B. pendula* progenies Velling (1985) stated that the within-population variation was as large as that between populations originating from latitudes  $60 - 63^\circ$  N. Raulo and Koski (1977) found that intra-group variation within provenances and between individuals was large compared to variation between stands or localities. Birch stands are not closed populations and gene exchange between stands is successful as a result of pollen dispersal by wind. A large within-population variation was also reported, for example, in two separate studies (Johnsson 1951 and Langhammer 1982, cited by Eriksson and Jonsson 1986).

### *Differences in qualitative traits; marker techniques*

Raulo and Koski (1977) reported a large variation in growth and stem quality between progenies from different plus trees. Many progenies attained a stem volume that exceeded the mean value of the test by more than 40 %, and good progenies could be found among both open-pollinated and cross-pollinated families. Variation in wood density is generally smaller than that in growth and stem quality characteristics, but significant differences can be found between progenies (Velling 1979a). Nepveu and Velling (1983) studied the inheritance in wood quality characteristics. The inheritance in basic density and shrinkage was fairly strong, but volume growth and pulp yield showed low heritability.

As attention has focused on the external quality of the stem for plywood production, no active breeding or selection of wood quality has so far been applied. The properties of birch pulpwood could be improved if the bark content could be reduced, the size and/or number of branches reduced and the carbohydrate content of the wood increased. Increasing the number of fibres without affecting the fibre length would improve the optical properties and bulk of the pulp. Tammisola *et al.* (1995) studied tree-to-tree variation to determine if there is variation in the pulpwood properties of *B. pendula* that can be used in breeding. They found that significant variation occurred between individual trees in the properties studied, and high significance levels give support for underlying genetic differences.

If DNA markers closely associated with pulping and papermaking properties were found, it would be possible to select the most desirable trees within a progeny more quickly and cheaply compared to the more or less destructive analyses of mature trees (Tammisola *et al.* 1995). Altogether 157 nucleotide sequences and 177 proteins of *B. pendula* are listed in the database of NCBI. Molecular biology is the most active research area within biology and the number of known nucleotide sequences and proteins is increasing rapidly. The databases at <http://www.ncbi.nlm.nih.gov/> have the most updated information about genetic markers. For an old review on genetic markers in *Betula*, see (Hattemer *et al.* 1990).

### **C. Adaptivity to climatic conditions**

#### *Growth cessation and winter hardening*

In cool and temperate regions the annual temperature rhythm is the main regulating factor of the environment. Forest trees are adapted to the variation of the growing season between years as well as to the long-term average.

The timing of growth cessation and the subsequent process of winter hardening is determined by a joint effect of heat sum and night length (Koski and Sievänen 1985). In Punkaharju, Finland (61°48' N), growth ceased at the cumulative temperature sum of 800 degree-days and night length of 7.5h. Koski and Sievänen (1985) predicted that an adapted provenance of *B. pendula* will cease growth by the accumulation of about two-thirds of the total heat sum for a normal growing season. If northern provenances were moved southwards, growth would cease with two thirds of the original local heat sum, but due to longer nights, growth would cease a few days earlier, that is, with a smaller heat sum than at the original locality. A northern provenance from Punkaharju (Finland) moved to Suwalki (Poland) would cease growth three weeks earlier than the local one. The opposite transfer northwards of southern provenances would lead to growth cessation three weeks later than the local ones. The heat sum characteristic of southern provenances would not be reached until the beginning of September, but long nights would induce growth cessation 10 days earlier, thus adapting the trees for the local growing season (Koski and Sievänen 1985).

Breaking the dormant state is affected by chilling temperatures during winter. The effect of chilling is cumulative increasing up to a threshold when the buds are released from dormancy. Bud burst and growth

start in spring occur after the accumulation of a certain heat sum above a specific base temperature (Myking and Heide 1995).

### *Growth start*

As *B. pendula* is geographically widely distributed, it has by latitude and altitude determined ecotypes with different optimum and critical chilling temperatures and durations. The ecotypes of northern origin have the earliest bud burst with variation in the requirement for duration of chilling but not for chilling temperature. A longer chilling requirement is found in southern ecotypes. A clinal difference in the base temperature for growth among the ecotypes is also found. In the studies of Myking and Heide (1995) the north Norwegian ecotypes flushed 2.5 months earlier and developed faster and had a lower base temperature for growth than ecotypes from southern Scandinavia. The Danish birches have adapted to a milder and more variable winter climate by developing greater dormancy stability involving both a longer chilling requirement and a higher base temperature. Night length had no effect on bud burst after the chilling requirement was fully met. After full dormancy release, time of bud burst in birch depends solely on the temperature regime in late winter and spring (Myking and Heide 1995).

The upper temperature limit for normal dormancy release in birch is probably slightly above 12°C. Chilling deficit is thus unlikely to occur in Scandinavia and in other areas where the chilling requirement is far exceeded, even with a climatic warming of 7 – 8°C above the current normal winter temperature. The likely effects of a climatic warming include earlier bud burst, a longer growing season and increased risk of spring frost injury, especially in northern ecotypes (Myking and Heide 1995).

### *Growth capacity of different provenances*

In provenance trials with *B. pendula* at four sites within the latitudinal range of 56 – 64°, Johnsson, 1977 (cited by Eriksson and Jonsson 1986) reported that long-distance transfers both southwards and northwards tend to result in growth reduction. The longer the transfer northwards, the more is the growth period prolonged for the material from the south and the higher is the risk for dieback of the leaders. Short-distance transfers (< 250 km) do not influence the height growth considerably. Kleinschmit and Svolba (1982) reported on three-year heights of populations from central Europe to Finland. The Finnish and Swedish populations moved to Germany performed poorly as a consequence of the long-distance transfer southwards. In a study of growth of seedlings (Velling 1979b) a dependence between height and latitude of the origins was found. Seedlings of different provenances (from Latvia, 56°31'N, to central Finland, 61°48'N) were grown in central Finland and the more southern the origin, the greater the height of seedlings. A correlation was also found between the degree of leaf yellowing and the origin of seedlings. While the Finnish origins had turned completely yellow in the autumn, the Latvian origins still retained their green colour. The colouring was connected with survival, with poor winter resistance causing increased mortality in the southern origins.

## **7. Ecology and Physiology**

### **A. Dynamics of regeneration**

Birch is a pioneer species, and it quickly colonises bare areas and does not tolerate shading. Young birches can not survive fire because of their thin bark, but some mature trees may, because the thin forest floor under birches can not support intense and persistent surface fires. Post-fire pioneer successions are often dominated by birch. Abundant seed production enhances the pioneering character of birch. Without fire or human intervention birches would be replaced in succession by more shade-tolerant and longer-lived species (Perala and Alm 1990).

### Light

Seedling density is independent of canopy cover, *i.e.* germination is unaffected by light, but the ability of birch seedlings to penetrate the canopy is low and they cannot establish in even the lowest vegetation. Most birch seedlings are only about 5–12 cm tall after the first year, and competing vegetation on fertile sites can easily overgrow and subdue them. The birches are also sensitive to chemical interference (allelopathy) by other plants (Perala and Alm 1990). Moreover, shaded birches are a preferred host by insect herbivores (see *e.g.* Ruohomäki *et al.* 1996).

Shading by neighbouring trees has a profound effect on shoot growth. In a short-term experiment shaded seedlings were higher than those reared in the simulated sun-light (Aphalo and Lehto 1997). Fewer buds are initiated and a higher proportion die in zones of heaviest shading (Atkinson 1992). Optimum sunlight for silver birch height growth was found to be 43 % of full sunlight for weeded seedlings, but 24 % for seedlings competing with weeds. Silver birch has also been found to grow less when sunlight decreases from 56 to 16 %. In greenhouse experiments with conifers, birch was more sensitive to both its own canopy and root competition than to competition by conifers (Perala and Alm 1990).

The Eurasian birches can endure as much as 90 % shade by adapting leaf structure. As sunlight diminishes, the light intensity for photosynthetic saturation, maximum photosynthetic rate, leaf mesophyll thickness, and chlorophyll concentration all diminish. The maximum photosynthetic efficiency for silver birch is at 10–50 % full sunlight, much higher than for shade-tolerant plants (Perala and Alm 1990).

### Temperature

The birches are adapted to cool climate and grow best at about room temperature. Once the soil temperature reaches 2–3°C, the growth of silver birch depends more on air temperature than on soil temperature. Seedling shoots grow in direct proportion to heat sums, gradually diminishing as photoperiod shortens. New shoots can tolerate growing season temperatures of -3 to -5°C (Perala and Alm 1990).

### Water requirements

Assimilation of silver birch is fastest at about  $-5 \times 10^2$  kPa and water use diminishes in wet conditions. The birches are sensitive to both drought and flooding. Seedling mortality increases at water potentials below  $-1.6 \times 10^2$  kPa. However, adaptability of birches to anaerobic conditions by oxygen transfer from the shoots to the roots, reduces the effects of flooding. Fertilisation improves water uptake and drought resistance (Perala and Alm 1990).

The birches use water inefficiently. Silver birch seedlings maintain turgor at high soil water potential only by closing stomata, which partially close at about  $-15 \times 10^2$  kPa. In large trees daily transpiration per unit of foliage mass is about 514 kg water/kg foliage. Extreme transpiration demand reduces growth even on moist soil because transpiration and photosynthesis have partly separate control systems. Drought depresses photosynthesis more than it does transpiration (Perala and Alm 1990).

### Nutrients

To achieve maximum productivity, white birch requires all necessary nutrients, an optimum ratio of nitrogen sources  $\text{NO}_3^-$  and  $\text{NH}_4^+$  in a rhizosphere and an optimal total nutrient solution (Ingestad 1971). In general, *B. pendula* is a nitrogen-limited species. Low-nitrogen conditions reduce growth and increase amount of condensed tannins, whereas in high nitrogen conditions *B. pendula* grows faster and the content of flavonoids in foliage is higher (Keinänen *et al.* 1999). The effects of nitrogen fertilisation on herbivore resistance are not clear. Nitrogen fertilisation did not affect preference of mammalian herbivores on *B. pendula* seedlings. On the other hand, the autumnal moth grew larger on fertilised seedlings (Mutikainen *et*

al. 2000). The limiting effects of other nutrients on the birches are not as well defined. At optimum levels, both zinc and manganese stimulate seedling growth, but toxicity problems have also been reported. There is a wide genetic variation in zinc tolerance and uptake. *B. pendula* has a high requirement for sulphur and it readily takes up boron. It sometimes suffers from manganese toxicity on poorly drained peat. The pH optimum for *B. pendula* and *B. pubescens* is between 4 and 5. *B. pendula* can tolerate some soil salinity (Perala and Alm 1990).

### *Effects on soil*

*B. pendula* grows on fertile mineral soils and on drier and lighter soils than *B. pubescens*, which grows commonly on both peat and mineral soils (Gimingham 1984). The birches usually improve soils by efficiently cycling nutrients. First generation birch stands on former *Calluna vulgaris* (heather) heathland have increased earthworm activity, higher soil pH, greater total P, higher base status, faster rates of N mineralisation and cellulose decomposition and more diverse ground flora. Surface soil N, P, K, Ca, Mg and Mn is increased. Silver birch on Sphagnum peat soils accelerates microbial decomposition, accumulates dead woody roots and increases soil bulk density (Perala and Alm 1990).

### *Photosynthesis*

CO<sub>2</sub> exchange variables showed considerable genetical variance in *B. pendula*. 54 % of variation in net photosynthesis, 36 % of variation in stomatal conductance and 45 % of variance in intercellular CO<sub>2</sub> were assigned to family (Wang *et al.* 1995). Photosynthesis related traits are modified by environmental factors as well. Defoliation reduces leaf area and biomass, but not photosynthesis, since *B. pendula* is able to compensate the damage by increasing photosynthetic activity. In fact, in nitrogen-rich environments damaged leaves have higher photosynthetic activity than the undamaged controls (Ovaska *et al.* 1993). The degree of compensation is dependent on the source of damage. The compensative response of *B. pendula* after artificial damage does not differ from that of the autumnal moth (Ovaska 1993), whereas the response was relatively weak after the damage by the alder beetle *Agelastica alni* (Oleksyn *et al.* 1998).

### *Root development*

Birch trees exploit soils efficiently by developing both an extensive and dense surface root system to intercept precipitation and sinker roots to penetrate dense 'pans' and exploit deep water. The tap-root becomes horizontal after about 30 cm or is overtaken by side roots. Narrower roots may penetrate to a considerable depth. The extreme length of horizontal roots can be 25 m on sandy soils. In anaerobic conditions, *B. pendula* and *B. pubescens* roots elongate more than in aerobic conditions. Birch roots penetrate deep into poorly drained soils such as peats, and benefit inter-grown conifers by oxygenating the soil. On the other hand, birch small-root biomass may be twice that of the conifers offering intense root competition (Perala and Alm 1990).

### **B. Mycorrhizae**

Early mycorrhizal infection is an important factor in the successful establishment of birch seedlings especially on nutrient-poor soils. The rates of nitrogen mineralisation of many forest litters are so slow that nitrogen can become the key growth limiting element. Some ectomycorrhizal fungi (e.g. *Amanita*, *Boletus*, *Paxillus*, *Suillus* and *Thelephora*) have proteolytic activity and thus the potential to mobilise nitrogen from proteins, peptides and amino acids and make it available to the plant. Some fungi, like *Laccaria laccata*, lack this activity and are dependent upon mineralisation processes initiated by other organisms (Read 1991). The assimilation of mycorrhizal amino compounds also provides supplementary carbon as well as nitrogen to the host plant. Abuzinadah and Read (1989) showed that up to 9 % of the carbon assimilated by the host plant (*B. pendula*) over a period of 55 days was derived heterotrophically from the protein by

mycorrhizal fungi. This is thought to be an adaptation to shade stress in young trees which spend the early part of their lives under the canopy of mature trees and which need to supplement their carbon budgets by heterotrophic assimilation. Grellier *et al.* (1984) showed that association with a mycorrhizal fungus (*Paxillus involutus*) practically doubled the growth of *in vitro* grown birch seedlings compared to non-mycorrhizal seedlings. Soil fauna have also been found to positively influence nutrient uptake and net production of birch seedlings (Setälä and Huhta 1991). Mycorrhizae can be inhibited by acute phosphorous deficiency, but can be encouraged even by a light application of phosphorous. Mycorrhizae like *Paxillus involutus* increase zinc tolerance by adsorbing zinc to the hyphae and slowing its transport to the shoots (Perala and Alm 1990).

Because little growth is possible on the nutrient reserves available in the seed, the seedlings are dependent on an external nutrient supply by the production of the first pair of leaves. Newton and Pigott (1991) report that ectomycorrhizal infection was indeed recorded by the time the first pair of leaves had expanded. Two groups of mycorrhizal fungi have been recognised: "early-stage" fungi (*Inocybe* spp., *Hebeloma* spp. and *Laccaria* spp.) can inoculate roots of seedlings from basidiospores or from added inoculum whereas "late-stage" fungi (e.g. *Lactarius pubescens* and *Leccinum roseofractum*) infect by hyphal connection. In natural circumstances the "late-stage" fungi are the most vigorous colonists of birch seedlings (Read 1991).

### C. Diseases

#### *Firm Rot*

Black coloured firm rot around the pith is a serious cause of concern in young cultivated birch stands. Several different pathogens (fungi and bacteria) are responsible for the defect. Their entrance is, obviously, facilitated by frost cracks, insect exit holes (possibly the exit holes of *P. betulae*) and especially vole or moose browsing. The defect is especially harmful in plywood industry if the black colouring spreads outside the peeler core (Uotila 1987).

#### *Birch rust*

Birch rust (*Melampsorium betulinum*) is the most common leaf disease of *B. pendula*. The rust causes yellowing and premature falling of the leaves, but it does not affect buds or the wood. If epidemics appear in several consecutive years, the growth of young seedlings can be decreased because of the shortened assimilation period (Uotila 1987; Vuorinen 1992). There are clear genetic differences in susceptibility to rust among birch clones, and screening clones for resistance could be used in breeding. A leaf-disc bioassay was used for determining the field rust resistance of birch clones in the study of Poteri and Rousi (1996).

#### *Stem spotting*

Stem spot disease caused by a group of fungi (*Godronia multispora*, *Botrytis cinerea*, *Fusarium avenaceum*, *Cylindrocarpon* sp., *Alternaria* sp.) is common in nurseries and can be very detrimental to young birch seedlings. The small necrotic lesions in the bark produced in the autumn enlarge during winter, and seedlings may die before spring. These fungi infect trees through mechanical wounding sites, frost cracks or insect (especially cicada, *Cicadella viridis*) wounding sites (Juutinen *et al.* 1976). Stem spotting is common in birch trees grown in unsuitable sites, e.g. in waterlogged soils (Uotila 1987).

#### *Rot fungi*

*B. pendula* is mainly rotted by *Polyporaceae* fungi, which start the rotting in the heartwood in the inner part of the trunk, and then proceed to the living sapwood. Especially timber is susceptible to decay.

*Fomes fomentarius* and *Inonotus obliquus* are the most common rot fungi and they attack living trees through wounds. *Ochroporus igniarius* also infects living trees.

*Piptoporus betulinus* is less common and attacks old or dead trees, destroying both heartwood and sapwood at the same time. The best way to prevent rot damage of living trees is good forestry practice: thinning should be done in time and only dead branches pruned, since branch scars act as a route for rotting fungi (Uotila 1987). Other fungi that rot dead trees and timber are *Stereum sanguinolentum*, *S. purpureum*, *Pycnoporus cinnabarinus*, *Cerrena unicolor*, *Trametes multicolor* and *T. hirsuta* (Uotila 1987).

#### D. Insect herbivory

Insect herbivory affects birch growth. For instance, in 1965 – 1967 the autumnal moth (*Epirrita autumnata*) defoliated over 5000 km<sup>2</sup> of birch forest in Finnish Lapland (Lehtonen and Heikkinen 1995). On the other hand, birches have a large community of invertebrate herbivores (Annala 1987), which may damage seedlings and trees locally, but at the stand scale birch is able to compensate the damage. Of geometrid defoliators living on *B. pendula*, *Opheroptera brumata* is among the most voracious species (Tikkanen *et al.* 1998, 1999, 2000). The species is common in Baltic countries, southern Scandinavia and central Europe. The damage may be severe after the insect outbreak but usually climatic conditions enable birch to compensate the damage by re-growth. Beetles may damage trunks of mature trees, and thereby reduce the economical value of the timber. For instance, *Hylecoetus dermestoides* and *Trypodendron signatum* bore tunnels in logs and timber. *H. dermestoides* can attack also living trees and a simultaneous infection by rot fungi can be fatal to the tree (Raulo 1981). Larvae of the birch cambium fly, *Phytobia betulae*, mine in the birch wood near the cambium layer and cause 1 – 4 mm wide brown streaks in the wood. The streaks decrease the value of birch wood used in plywood and furniture industry. The damage is aesthetic; the mechanical strength of the wood is not affected. The resistance mechanisms of birch and the biology of *Phytobia betulae* are not known. Ylioja *et al.* (1995) studied the susceptibility of European (*B. pendula*) and Japanese white birch (*B. platyphylla*) to *Phytobia* damage. Birch progenies which had *B. platyphylla* in their ancestry included more pith flecks than pure *B. pendula* progenies. Fast growing birches were also more susceptible to *Phytobia* attack. Differences in susceptibility to *Phytobia* attack between birch clones and progenies of plus trees could be used in resistance breeding.

Besides the genuine seasonal change in foliar phenolics (Salminen *et al.* 2002), insect grazing or artificial damage increases the level of phenolics in the leaves of *B. pendula*. In the study of Hartley (1988), increases in phenolic compounds did not affect further feeding, either by a natural birch-feeding herbivore (*Apocheima pilosaria*) or a polyphagous non-birch feeding insect (*Spodoptera littoralis*). Hence, there is no evidence that the tree's responses were specific defences against further attack by insect herbivores.

#### E. Mammalian herbivores

In Scandinavia, trees of the genus *Betula* are important winter food for herbivorous animals, especially voles (*Microtus*, *Clethrionomys*), hares (*Lepus*) and moose (*Alces alces*) (Rousi *et al.* 1989, 1990; Jia *et al.* 1997). They usually destroy woody plants in wintertime when alternative food plants are under snow cover. The variation of resistance among origins and families and even among individual seedlings within a genus can be very large. The centres of origin of cultivated plants are thought to be the best places to find resistances to diseases and herbivores. In these centres, plants have been exposed to selective pressure from local pathogens and herbivores for a long time, and have consequently developed resistance to them. Bryant *et al.* (1989) indicated that *Betula* and *Salix* species from Pleistocene refuges (Alaska and Siberia) were more resistant to mountain hare than species from regions that were glaciated during the Pleistocene. Likewise, birches from Iceland, where there were no browsing mammals before the

Norse colonisation, were more susceptible than birches from regions with more browsing mammals, that is, Alaska, Siberia and Finland (Bryant *et al.* 1989).

The bark of seedlings, young shoots and twigs of *B. pendula* contain resin droplets that consist of papyriferic acid and other triterpenoids. The juvenile resistance is accounted for by the resin, which is synthesised in and excreted by glands that are active only during the season when the primary apical growth of the shoot takes place (Taipale *et al.* 1993). On the other hand, phenolic substances are present in winter-dormant birches of all growth stages, but are rapidly metabolised by the plant in the spring when leaves emerge. Of these, platyphylloside is shown to exhibit repellent and anti-nutritional effects in mountain hares (Palo *et al.* 1992).

In *B. pendula* there is a sharp decrease of resistance after the tree has reached certain dimensions and the tree is no longer within reach of the herbivores. In the feeding trials with hare, Rousi *et al.* (1989) found that 1-year-old seedlings were less palatable than twigs taken from 7-year-old saplings of the same origin.

### Vole

Voles cause considerable damage to forest plantations, especially during the peak years of density fluctuations. Birch plantations are especially vulnerable, since they are often afforestations of old fields, which are habitats favoured by *Microtus* voles (Rousi *et al.* 1990). The vole destroys birch seedlings under snow cover and it can eat the bark of the seedlings until the basal diameter reaches 4 cm (when the seedling is about 5 years old). Triterpenes in young birch seedlings seem to be deterrents of vole feeding. However, as the resin droplets are mainly situated in the top parts of the seedlings, and voles usually feed at the base of the seedling, they avoid the deterrent substances of the resin droplets. In field tests of Rousi *et al.* (1990), there were no clear differences in resistance between European white birch families in field tests with voles, but the Japanese white birch (*B. platyphylla*) turned out to be especially resistant to vole feeding. Rousi *et al.* (1990) suggest that hybrids between *B. pendula* and *B. platyphylla*, or Finnish-Siberian crosses of *B. pendula*, could be used to increase vole resistance.

### Hare

Hares feed on the upper branches of young birch seedlings (of 40 – 70 cm) and especially in winter the damage can be fatal for the seedlings. Hares are discriminating feeders and determination of palatability is guided to a large extent by olfactory stimuli, and the resistance of young birch seedlings is tied to the production of papyriferic acid. For example, in the feeding experiments of Rousi *et al.* (1991), for the mountain hare (*Lepus timidus*) the palatability of birch seedlings and saplings was strongly and negatively correlated with the number of resin droplets on the bark. The Japanese white birch (*B. platyphylla*) turned out to be the most resistant of different birch species.

Rousi *et al.* (1991, 1996) also tested how the growth of birch correlates with resistance and whether fertilisation lowers the resistance of seedlings to browsing. Contrary to predictions of growth-defence trade-off theories, no trade-offs were found in the resistance and growth rate. Fertilisation stimulated growth but did not affect the palatability of the seedlings. Consequently, fast growing birch species and families should not be more susceptible to damage by herbivores, and fast growth can be promoted parallel with herbivore resistance by means of breeding.

### Moose

Moose browsing on young birch seedlings occurs throughout the year. During winter only twigs are browsed, but during summer both leaves and young twigs are browsed. In order to feed on the young twigs of the crown, moose often break the main stem of the saplings. Moose can cause serious damage to birch

plantations. In Finland, plantations established in 1976 – 1977 were studied in 1985 (Heikkilä and Raulo 1987). Only one third of the plantations were found to be undamaged. Half of the total area had been damaged slightly and 15 % seriously. At the time of the establishment, though, the moose density was very high, 3 – 8 animals/1 000 ha. With a density of 2 – 3 animals/1,000 ha moose are not a serious threat to growing birch. Also, establishing plantations close to built-up areas or main roads can considerably prevent the risk of moose damage.

Heikkilä *et al.* (1993) showed that after stem breakage, the recovery during the first two years was fairly good. If the stems were at the leader shoot of the previous year, re-growth was weaker than in unbroken trees, the angle of crookedness was stronger and wound healing weaker. Injuries to the wood commonly become discoloured, and there was discoloration in 80 % of the sample trees. The significance of discoloration and decay depends on their distance from the wood surface, and the effect of discoloration on the quality of logs, when used for saw timber or veneer, can only be determined after a longer growth period. Bergström and Danell (1987, 1995) simulated winter browsing and summer browsing of moose, and studied the effects on the morphology and biomass of *B. pendula*. The birches responded to simulated winter browsing by growing fewer but larger and more branched shoots. There was also a slight decrease in viable seed production (Bergström and Danell 1987). In the experiments of Danell and Huss-Danell (1985) birches browsed by moose had more ants, psyllids, leaf-galls, leaf-miners and other leaf-eating insects. The leaves of browsed trees were larger and heavier, appeared greener and contained more nitrogen and chlorophyll. A decrease in resin content was observed. The trees seemed to allocate most of the nutrients and energy to growth in order to grow above the browsing line (Danell and Huss-Danell 1985). No induced defence in juvenile trees has been found; those trees that had been browsed during the previous winter were more palatable than previously un-browsed trees (Danell *et al.* 1985). Defoliation (simulated summer browsing) resulted in an overall decline in biomass and reduction in height and diameter growth. The long-shoots produced on defoliated trees were smaller and suffered more from tip-drying than shoots on control trees (Bergström and Danell 1995).

## F. Abiotic damage

Abiotic damages are caused by too high ground water, drought, frosts and frost cracks (Uotila 1987). Snow load can cause stem breakage or permanent bending in young trees (Hanneliuss *et al.* 1989). Mechanical wounding increases fungal infections. UV-radiation does not seem to affect the growth, morphology or specific leaf area of *B. pendula* seedlings. The absorption by the secondary metabolites (phenolic glucoside, phenolic acids and flavonoids) provides the main part of the total UV absorbance of birch leaves. Plant secondary metabolism responds to enhanced UV-radiation by increasing synthesis of the above mentioned compounds that are the most effective UV-protectors (Lavola *et al.* 1997). The growth of *B. pendula* does not seem to be adversely affected by acid rain (Ashenden and Bell 1988). Instead, there was a stimulation in the height of birch seedlings with increasing acidity. A slight chlorosis of leaf margins after exposure to 2.5 pH rainfall was observed. Soil characteristics might have an influence on the sensitivity to acid rainfall.

Ozone causes physical damage on leaf surface, *i.e.* chlorosis, decoloration, black spots and necrotic areas and finally, leaf shedding (Maurer *et al.* 1997). Moreover, it changes the balance between CO<sub>2</sub> assimilation and stomatal conductance, which may severely limit plant's ability to repair ozone damage at the cellular level (Zhang *et al.* 2001). Ozone activates biosynthetic pathway, and thus the production of phytohormone ethylene (Kangasjärvi *et al.* 1997). Sequences of the cDNA of 1-aminocyclopropane-1-carboxylate oxidase (ACO), the enzyme catalysing the last step in ethylene biosynthetic pathway, as well other possible ACO-homologue fragments have been submitted to the Plant Gene Register (EMBL accession numbers X97993, X97992, X97994 and Y10749). Interestingly, *B. pendula* is among the most ozone-resistant woody plants in central Europe. The gas exchange traits differ by factor or two when compared to more vulnerable woody plants (Zhang *et al.* 2001).

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### SECTION 3

## NORWAY SPRUCE (*PICEA ABIES* (L.) KARST)

### 1. General Description and Use in Forestry

#### A. Taxonomy

Norway spruce (*Picea abies* (L.) Karst) belongs to the genus *Picea*, which includes at least 36 different species that all have their distribution in the northern hemisphere (Schmidt-Vogt 1977). The species *P. abies* has been designated by a large number of synonyms, partly caused by classification to different species or subspecies due to its great number of varieties and forms. The best known of these is the classification of the Siberian spruce in a separate species, *P. obovata*, based on the shape of its cone scales. These variations, however, should be considered as normal patterns of variation within a widespread species and should at most be used to designate different climatic varieties (Schmidt-Vogt 1977, 1978).

#### B. Use of Norway spruce

Norway spruce is the economically most important conifer tree species in Europe. It has a long history of cultivation in central Europe, and has been seeded and planted very intensely since the middle of the 19th century. This has changed natural forests into artificial ones and has led to the species' introduction far outside its natural range, both in countries where it occurs naturally (e.g. Germany and Norway) and in new countries such as Denmark, Belgium and Ireland. To some extent, Norway spruce has also been planted in North America.

Norway spruce has shown good yield and quality performance under very different site conditions, and this favoured the species over a long period. In some areas, where maladapted provenances were used, damage and reduced yield have occurred. In the last two decades the species has suffered severely during the forest decline in central Europe, resulting in stands with high percentages of trees with needle loss (Wolf 1995) or in completely destroyed stands (Vancura 1995). The health problems of the central European spruce forest, and reduced possibilities for recreation in young spruce stands, have to some extent reduced the popularity of this species for reforestation, particularly outside its natural range.

### 2. Forest Practices for Norway Spruce

#### A. Breeding of Norway spruce

Usually the goal of breeding programmes is to produce superior material for reforestation by managing genetic variability. Breeding of Norway spruce was initiated in several European countries in the late 1940s (Danell 1991, Mikola 1993). In some countries the work started with the selection of phenotypically superior trees ("plus trees") in natural stands (Skroppa 1982b, Mikola 1993, Gabrilavicius and Pluura 1993). Mature trees that had superior height and diameter growth, and stem and branch quality, compared to neighbouring trees in the stand were selected. They were grafted in multiple copies on rootstocks in clonal archives or seed orchards. Each grafted seed orchard is composed of a rather large number of selected clones (50-500), with the intention of seed production for one geographic region. The

seed orchard will generally start to flower 10-15 years after grafting. However, both the regularity and amount of flowering are very dependent on climatic conditions at the orchard site. To promote flowering, seed orchards have often been located to sites with a warmer climate than that from which the selected parents originate and where the orchard seed is intended for use.

It was soon realised that the selection of plus trees in natural Norway spruce stands is not an efficient method of selecting superior genotypes. It is necessary to test the genetic value of each parent, based on an evaluation of its offspring. In Norway this is done in progeny tests planted at several sites, where measurements are made of survival, height and diameter growth, and quality traits. The progeny tests are sometimes supplemented with tests in which seedlings are grown under controlled conditions in growth chambers and measurements are made of physiological traits. On the basis of several traits, a subset of the original parents is selected for further breeding. Seeds for practical planting can be collected in the orchard from the selected parents, the orchard can be thinned, or a new orchard can be established based on the selected group.

In other countries, breeding programmes were based on selected materials from populations with high adaptive potentials observed in comparative provenance trials. According to performances measured in progeny trials with families from these populations, the best individuals within progeny were selected either directly to produce seeds in seed orchards, or to create a breeding population through controlled crosses. Some of these programmes were also aimed at mass production of rooted cuttings of tested and selected clones (Biro 1982, van de Sype and Roman-Amat 1989, Kleinschmit 1993). Of major concern in the development of breeding strategies have been the breeding objectives; the sizes of the breeding and seed producing populations required to maintain genetic diversity; design and efficiency in testing; and identification of suitable regions where the orchard seed should be recommended for use. Based on test results, gains from the original plus tree selection have been estimated, at 6% genetic improvement in height growth at stand closure (Danell 1991). An additional 10% advance in the selected trait can be expected after progeny testing and further selection.

In Norway, Sweden and Finland several thousand selected spruce trees have been grafted in seed orchards or clonal archives. A large number of these have been progeny tested. Most seed orchards, however, consist of the original set of non-tested parents. In some orchards selective harvests have been made on the basis of progeny test results. The main selection criteria have been traits characterising the annual growth rhythm, height growth, and the avoidance of damage in field tests. Artificial freezing tests are being used to determine autumn frost hardiness, particularly for materials intended to be planted at high altitudes or far north. Each seed orchard is intended to produce seed for a specific region, which is determined by the origin of the parents and the growth rhythm and hardiness performance of the orchard offspring.

Seed orchard breeding is also the most common technique being used for spruce in central and eastern Europe (e.g. Dolgikh 1993, Gabrilavicius and Pliura 1993, Kleinschmit 1993). Special types of orchards have been established, based on materials from provenance trials, in order to reconstitute provenances that probably no longer exist or are untraceable (Giertych 1993). Clonal breeding programmes based on rooted cuttings have been developed in Germany (Kleinschmit 1993) and Sweden (Karlsson 1993).

## **B. Reproductive methods**

### ***Flower induction***

Application of growth regulators, primarily gibberellins, has become an efficient method of regulating flowering in forest trees. In the case of Norway spruce grafts, treatments during the shoot growth period (with gibberellic acid alone and in combination with heat) have been efficient in promoting female

flowering (Johnsen *et al.* 1994). Heat treatment alone will induce male flowering. Of mechanical flower stimulation techniques attempted in Norway spruce seed orchards, only thinning is useful on a larger scale (Schneck *et al.* 1995). The problem of shortening the juvenile phase and obtaining regular flowering at an early age remains unsolved (Chalupka 1991).

### *Vegetative propagation*

Norway spruce has for decades been propagated by grafting. Scions to be grafted are taken from the crown of mature trees and grafted onto a rootstock, which is a young spruce seedling. This technique is being used to establish clonal seed orchards and clonal archives.

Vegetative propagation with rooted cuttings is easily achieved with young spruce seedlings and can be applied on a large scale (Kleinschmit *et al.* 1973). The propagation can be made either in early spring before bud flushing, or after shoot growth terminates at the end of the summer. Both the rooting capacity and the growth form of the cutting depend on the age of the cutting donor, but they can be improved in a proper rooting environment. A large genetic variation has been observed in rooting capacity and subsequent growth habit of the cutting (Johnsen and Skråpø 1992). Several systems of propagation have been developed in order to keep the plant material at a juvenile stage (Roulund 1981, Dekker-Robertson and Kleinschmit 1991). Clonal tests with rooted cuttings have provided valuable information for Norway spruce genetics research, in addition to providing clonal material with high genetic value intended for commercial use (*e.g.* van de Sype 1989, Kleinschmit and Svolba 1991, Isik *et al.* 1995).

Cell and tissue culture techniques for micropropagation have recently been developed for conifers. The most promising method for Norway spruce appears to be somatic embryogenesis, obtained for the first time by Hakman *et al.* (1985). Somatic cells are stimulated to develop into somatic embryos, somewhat analogous to that of a zygotic embryo. The somatic embryo can be stimulated to mature and develop into a plant which will be of the same genotype as the zygotic embryo used for induction of this embryogenic cell line (Hakman 1993, von Arnold *et al.* 1995, von Arnold 1996, Egersdøttir and von Arnold 1998). However, in *P. abies* somatic embryogenesis has also been initiated from explants other than zygotic embryos (Wescott 1994, Ruud 1993). The somatic embryos can be propagated in unlimited numbers on a large scale and can be formed as artificial seeds. The method works only for a limited number of genotypes and requires controlling plant quality. Therefore, further developments are needed before somatic embryogenesis can be used for practical forestry purposes (Mo 1993). Norway spruce also belongs to the few spruce species with advanced *in vitro* regeneration, and thus could have possibilities for genetic transformation. There has recently been a breakthrough in production of stably transformed Norway spruce, based on *Agrobacterium tumefaciens* mediated transformation (Wenck *et al.* 1997) and on biolistic transformation (Walter *et al.* 1997). Also, genetic transformation of Norway spruce pollen and the use of transformed pollen in controlled pollinations is in progress (Hägglund *et al.* 1997).

### **C. Reproductive materials used**

The regeneration of Norway spruce forests is based on both natural regeneration and planting, with an emphasis in many countries on natural regeneration where that is a feasible method. The largest proportion of Norway spruce seeds being used world-wide are collected in natural or planted stands. Each seed lot is identified by the geographic origin of the stand. In several countries it is required that the seed stand be selected for superior performance (EEC 1966, Muhs 1986). The relative amounts of stand and seed orchard seed being used vary considerably between countries, and between regions within countries. Clonal forestry based on rooted cuttings, which was initiated to some extent in the 1970s in Germany and Sweden, occurs at present only on a small scale.

In the Nordic countries, stand seed accounts for the largest proportion of seed used. The percentages of stand seed used in 1994 were: 89% (Denmark), 84% (Sweden), 81% (Norway) and 80% (Finland) (personal communication Bjerne Ditlefsen, Lennart Ackzell, Gunnar Haug, Hannu Kukkonen). The percentage from seed orchards varied between 10 and 20%. Sweden is the greatest importer of spruce seed, 35% of which is stand seed. Rooted cuttings account for less than 1% of the total number of Norway spruce plants.

#### **D. Provenance transfers**

The first provenance transfers were more or less random and unrecorded, with the result that in large areas of central Europe and southern Scandinavia most spruce stands are of unknown and mixed origins. Subsequently, provenance trials have provided information about the effects of transferring provenances from different parts of the range of the species and have identified provenance areas which generally have both a high adaptability potential and high growth capacity. Examples are provenances from southern Poland and Romania, which have been planted with success in several countries (Lacaze 1969, Krutzsch 1992, van de Sype 1998). Some other provenances present a low adaptation potential and exhibit huge variations in performance for survival rate and growth ability according to trial location (e.g. van de Sype 1998).

Provenance transfers have been used deliberately in several cases where materials with specific adaptive properties were sought. An example is the transfer of late-flushing Norway spruce provenances from Byelorussia and the Baltic countries to sites exposed to late spring frosts in southern Sweden. These provenance transfers have reduced frost damage and improved growth (Werner *et al.* 1991, Werner and Danell 1993). In other cases, however, maladaptation has occurred, particularly after transfers of provenances from a warm to a colder climate. In south-eastern Norway, spruce provenances from Austria and southern Germany were extensively planted during a 20-year period starting in the 1950s. A survey of 79 planted stands of central European origin and 21 of local origin, all at the age of 27 years, was recently made in Østfold County, Norway (Skroppa *et al.* 1993). In the stands of local origin, 30% of trees were classified as having saw timber qualities, while only 7% of those in the central European stands obtained the same quality classification. This provenance transfer generally had a negative effect on timber quality and a small positive effect on volume production, even if a few of the stands planted with southern provenances had both high volume production and good stem quality. It is not known to what extent the pollen cloud from these stands may cause reduced fitness in offspring from the seed in neighbouring stands of local origin.

#### **E. Conservation of Norway spruce genetic resources**

In many countries the combination of natural regeneration and planting of materials with a broad genetic diversity is the basis for conservation of genetic resources of Norway spruce. In addition, several specific conservation measures are taken. *In situ* activities include national parks, nature reserves and specific conservation or gene reserve forests. The grafted clone banks and seed orchards and provenance and progeny tests constitute the *ex situ* gene conservation, together with long-term seed storage. Co-operation at the European level is achieved through the recently established European Forest Genetic Resources Programme (EUFORGEN). Norway spruce is one of the species for which a co-operative network has been formed (Turok *et al.* 1995, Turok and Koski 1997, Koski *et al.* 1997).

### 3. Centres of Origin and Immigration History

#### A. Natural distribution and origin

The natural distribution of *P. abies* can, according to Schmidt-Vogt (1977), be divided into three areas: the central and south-eastern European, north-eastern European and Siberian spruce areas. The first covers mainly mountainous and sub-alpine regions and is separated from the second by a spruceless area in Poland. The north-eastern European area includes the Baltic, Nordic and Russian spruce. The Siberian area covers all spruce east of the Urals.

The total natural distribution of *P. abies* covers 31 degrees of latitude from the Balkan Peninsula (latitude 41°27'N) to its northernmost extension near the Chatanga River, Siberia (latitude 72°15'N). Longitudinal range is from 5°27'E in the French Alps to 154°E at the Sea of Okhotsk in Eastern Siberia. The vertical distribution is from sea level to altitudes above 2 300 m in the Italian Alps. Outside this area the species has been widely planted, particularly in central Europe (Ellenberg 1988) and in Scandinavia.

It is thought likely that *P. abies* had its prehistoric origin in east Asia, from which it migrated to Europe through Siberia and the Urals (Schmidt-Vogt 1977). During the last Ice Age, the species is assumed to have survived in refugia in four European regions: north-central Russia, the Carpathians and Transylvanian Alps, the Dinaric Alps and the Apennine peninsula. From these refugia, *P. abies* migrated to its present natural areas. The central and south-eastern European spruce originate from the last three refugia, while the north-eastern European spruce migrated from Russia.

In central Europe, *P. abies* could probably be found only in two small areas in the present Czech Republic about 11,500 BC (Vancura 1995). It migrated from the Beskids over the Sudeten to the Ore Mountains in Germany, which were reached about 6500 BC. Spruce established in the Harz Mountains about 4000 BC, while the Black Forest in south-western Germany was reached about 1500 BC. In Switzerland, Norway spruce established in the eastern and south-eastern part of the country about 8000 years ago and expanded to the valleys of the Alps within 3500 years (Bonfils and Sperisen 1997). The expansion was much slower North of the Alps.

The migration of Norway spruce to Fennoscandia took place from the Russian refugium. It advanced through the forest area at the present border between Russia and Finland and passed through Finland and northern Sweden into Norway in the period 3500-500 BC (Moe 1970, Schmidt-Vogt 1977, Hafsten 1991, 1992a, b). The advance was quite rapid; it has been estimated at an average speed of 9 km every ten years (Moe 1970).

### 4. Reproductive Biology

#### A. Sexual reproduction

*P. abies* is monoecious, having both male and female flowers on the same individuals but on separate organs. The male flowers are in most cases located at the base of the preceding year's shoot, while the female strobili are at the tip of the shoot, directed upwards. The reproductive buds are initiated during the growth season the year before.

Male meiosis and pollen development occur after the winter dormancy in late winter or early spring and are regulated by the temperature conditions (Luomajoki 1993). A male flower may contain as many as 600 000 pollen grains, which are released at anthesis and dispersed by wind.

Female meiosis usually starts before the female strobili become receptive (Sarvas 1968) and occurs close to or during the period of pollination. Pollen grains are accommodated in a pollen chamber which has

a limited volume and, on average, contains three to five pollen grains. They germinate and pollen tubes grow towards the female gametophyte (egg cell) in the ovule, where fusion of the male and female gametes takes place. Under natural conditions this normally occurs five to six weeks after pollination. The fertilised egg develops rapidly into a seed. Several egg cells may be fertilised in each ovule, but only one will develop into a seed. Empty seeds may result from lack of pollination or from abortion.

Climatic conditions play an important role during several stages of the reproductive process. High temperature during the growth season is one of the main factors favouring floral initiation and the development of reproductive buds which will flower the next spring. Dry conditions and moderate to high temperatures during the flowering period the following year are necessary in order to obtain sufficient pollination. Seed development and maturation require high accumulated temperature sums during the summer and early autumn. Such specific weather patterns during two successive years occur rather seldom, particularly far north and at high latitude regions, which may explain why seed crops are both rare and irregular in these areas.

### **B. Mating system and gene flow**

The mating system of Norway spruce falls into the mixed mating category. That is, the largest part of the seeds are produced through cross-fertilisation and the rest through self-fertilisation. Cross-fertilisations are both between trees that are close together in a stand, and among distant individuals in the same or nearby stands.

Spruce pollen is able to move over long distances. Andersson (1955) found that the amount of pollen at 2 500 m distance was 47% of the amount at the edge of the forest in one stand, and 15% in another stand. In two Norway spruce stands in Finland, Koski (1970) estimated the background pollination to be approximately 60% of the total pollen catch. Lindgren *et al.* (1991) reported results from studies of the dispersion of Norway spruce pollen artificially released in seed orchards outside the local pollen shedding season. These results showed a rapid drop-off in the pollen dispersion curve over distance from the source, with the largest amount of pollen being deposited less than 50 meters from the pollen source. However, large variation patterns most likely due to wind turbulence were found between pollen catches on individual days, indicating that a curve showing a smooth movement in the wind direction is not a good model for individual pollen dispersion events (Lindgren *et al.* 1991).

Both local and long-range pollen movements will determine the natural pollination patterns and actual gene flow of Norway spruce. On average, most pollinations will be with local pollen or pollen from nearby populations (Koski 1970), with exceptions in some years. In an allozyme marker genetic study in an experimental Norway spruce plantation, Xie and Knowles (1994) estimated the proportion of ovules fertilised with own pollen, pollen from trees within the plantation and that from trees outside the plantation to be 0.09, 0.75 and 0.16, respectively. The proportions varied considerably among parent trees. Wind-pollinated forest trees are known to have a high number of migrants per generation compared to other plant species (Govindaraju 1988, 1989). No good estimates of migration rates are available for Norway spruce, but Koski (1970) and Finkelday (1995) both concluded that the gene flow between nearby populations by means of pollen dispersal is effective and may play an essential role in the population genetics structure of Norway spruce.

The actual rate of self-fertilisations in natural populations may vary considerably between trees. It was found by Müller (1977) to vary between 7 and 18% in a study of five trees. Koski (1973) estimated the mean proportion of self-fertilisations at 10%, but concluded that only 1% of the filled seeds originate from self-fertilisations.

### C. Inbreeding depression

Most spruce trees will produce some filled seed after self-pollinations, but the seed yield is much reduced (Skårøppa and Tho 1990). The main reason for this is embryo abortions caused by lethal or deleterious genes that become harmful when they occur as recessive homozygotes in the selfed individuals (Koski 1971). Inbred spruce trees generally have reduced fitness compared to their outbred relatives. They have lower survival in the field, their growing season is shorter, and they grow more slowly (Langlet 1940, Eriksson *et al.* 1973, Skårøppa 1996). The inbreeding depression varies both among populations and among individuals within the same population (Skårøppa 1996). For a trait such as height growth at age ten years, it may vary in the range of 10 to 50% among selfed offspring of trees from the same population.

### D. The seed crop

Norway spruce trees undergo a rather long juvenile period, during which they will not flower and set seeds. In the open stand, sexual maturity will generally be reached after 20-30 years, while it occurs later in the closed stand (Schmidt-Vogt 1978). However, a substantial flowering and seed set may occur considerably earlier than 20 years from planting (Skårøppa, unpublished) if the temperature conditions are favourable for floral induction. In exceptional years, female flowering has been observed on eight- to ten-year-old and male flowering on twelve- to 15-year-old Norway spruce trees.

Under central European conditions, Norway spruce will flower several times in a decade (Schmidt-Vogt 1978). In the boreal forest, cone harvests occur less frequently and at irregular intervals. There were no cone crops in the lowlands of southern Norway between 1976 and 1983. Since then, however, substantial flowering and seed crops have occurred in 1983, 1987, 1989, 1993 and 1995. In northern Norway, successful seed harvests have been obtained only three times during the last 40 years (1958, 1970 and 1981).

In central European seed orchards, flowering has occurred less frequently than was expected (Kleinschmit 1993).

### E. Natural regeneration

The Norway spruce seeds are dispersed mainly by wind and partly by birds and animals (Sokolov *et al.* 1977). Most seeds will be dispersed close to the mother tree, but some may also be dispersed over larger distances.

Natural regeneration also very much depends on the species composition of the bottom and field layer. The most productive spruce forest appears to be the most problematic for natural regeneration, for example in "spruce forest with tall herbs" (*Melico-Piceetum aconitetosum*) and "spruce forest with tall ferns" (*Eu-Piceetum athyrietosum*). The optimal habitat for natural regeneration is the "spruce forest with small ferns" (*Eu-Piceetum dryopteridetosum*). Regeneration in our most common and widespread spruce community, "spruce forest with bilberry" (*Eu-Piceetum myrtilletosum*), is greatly hampered by a thick raw humus layer, especially at higher altitudes (Mork 1944, 1945, 1968).

The Norway spruce seedlings are very shade-tolerant and can survive for decades under a closed canopy (Siren 1955). They grow slowly during the first years, and the height growth increases after five to ten years (Sokolov *et al.* 1977, Nikolov and Helmisaari 1992). *P. abies* is often associated with grey alder (*Alnus incana*) on river plains in the boreal zone and may tolerate occasional flooding (Sokolov *et al.* 1977).

## F. Dynamics of regeneration

Boreal forests undisturbed by human activity have dynamic properties, with complex succession stages which influence habitats and create possibilities for regeneration. The most important natural disturbance factors are fire, storms, and pathogens such as bark beetles (Tømmerås 1994). The fire tolerance of *P. abies* is very poor (Drakenberg 1981). Mainly due to its shallow root system, spruce is intolerant to windthrow (Sokolov *et al.* 1977). Storms can blow down many trees, particularly in wind-exposed areas, where occasionally almost all the trees in a stand may blow down. Bark beetles (*Ips typographus*) can from time to time become a serious pest and kill trees (Christiansen and Bakke 1988). Together with gaps created by the death of individual old trees, these disturbances open up the canopy and lay the foundation for regeneration.

## G. Vegetative reproduction in nature

Under certain conditions, Norway spruce will naturally reproduce vegetatively through the lowest branches, which may come into contact with the soil and differentiate roots and new shoots (layering). This occurs particularly in alpine areas, where the climatic conditions prohibit sexual reproduction, and is therefore important for species distribution (Skoklefeldt 1993). Examples of such vegetative reproduction can be seen above the timberline where scattered clonal groups occur.

## 5. Crosses

### A. Crossability with other species

Crossability of spruce species can be judged both from introgressive hybridisation and from attempted controlled hybridisation. The only known natural hybrids involving Norway spruce are *P. abies* var. *obovata* with *P. jezoensis* and *P. koraiensis* (Schmidt-Vogt 1977) in eastern Asia. Successful artificial hybridisations are reported with eight other spruce species (*P. asperata*, *P. glauca*, *P. mariana*, *P. montigena*, *P. omorica*, *P. orientalis*, *P. rubens* and *P. sitchensis*) (Kleinschmit 1979). Rather few of the potential hybrid crossing combinations have been attempted.

### B. Interprovenance hybridisation

No crossing barriers are present between spruce trees from different provenances. Provenance hybrids are therefore produced naturally in regions with both indigenous populations and introduced provenances or cultivars. The extent and implications of these hybridisations depend on many factors, such as the characteristics of the provenances discussed in section VI.

Experimental results with inter- and intraprovenance crosses have demonstrated that the hybrids are intermediate between the two parents for most traits (*e.g.* Ekberg *et al.* 1982, 1991, Kaya and Lindgren 1992). Hybrid vigour therefore does not appear to be present to any large extent.

## 6. Genetic Variability

The number of chromosomes of Norway spruce is  $2n = 24$  (Schmidt-Vogt 1977 and references cited therein). Tetraploidy has occasionally been observed in Swedish and German provenances (Kiellander 1950) and has also been induced by colchicine treatments (Johnsson 1975). Trees with irregular chromosome numbers appear in general to have reduced fitness.

The genetic variability of Norway spruce has been studied using a large number of methods and at different genetic levels. More than 100 years ago, experiments replicated at several locations with seed lots from different origins (provenances) were carried out in Austria, Germany and Switzerland (Langlet 1971).

In these first genealogical experiments, traits such as height increment, needle morphology, growth habit, time of growth initiation and frost damage were studied. Later, both national and international provenance experiments were established, some of these organised by the International Union of Forest Research Organisations (IUFRO), and often including large numbers of provenances planted on multiple test sites in several countries (Kruttsch 1992). The traits studied are related, in particular, to forestry cultivation of Norway spruce. They characterise climatic adaptation, growth potential and quality. They are typically quantitative, having a continuous phenotypic distribution, and are strongly influenced by the environment. The same type of traits have been measured in numerous experiments with offspring from both natural and artificial populations, in most cases established with the intention of testing breeding materials. The field trials have been supplemented by tests under more controlled conditions, in which variability in specific physiological traits has been studied. Since biochemical markers became available, several population genetics studies have characterised the intra- and interpopulation variation and genetic structure of the species (e.g. Lagercrantz and Ryman 1990, Müller-Starck *et al.* 1992, Konnerth and Maurer 1995). Recently, molecular DNA marker techniques have been developed for Norway spruce (Bucci and Menozzi 1993, Binelli and Bucci 1994).

### A. Overall variability

Large genetic variability exists within the extensive range of the natural distribution of Norway spruce. The most pronounced adaptive patterns relate to populations' responses to climatic conditions. Across the European range of the species these patterns of variability can often be related to latitude and altitude of origin, and with degree of continentality, and will sometimes vary clinally. In central Europe, however, differences among populations from the same geographic region are in many cases large and reflect several generations of Norway spruce cultivation. This fact often blurs patterns of variation of adaptive traits. Traits that characterise the annual growth cycle, particularly onset of growth in the spring and termination of growth and development of frost hardiness in late summer, show the most pronounced provenance variability (Langlet 1960, Kruttsch 1975, Dormling 1973, Beuker 1994, Beuker *et al.* 1998). However, observations of these and of growth performance traits are made in common garden field tests comparing provenances that are transferred unequal distances. Such provenance transfers may affect provenances differently. Provenance differences must therefore always be interpreted relative to the planting site conditions.

Populations with an early growth start, often expressed as bud burst or bud flushing, originate from high latitudes in northern Scandinavia, Finland and Siberia and from high altitudes in the central European Alps (Langlet 1960, Kruttsch 1975, Holzer 1993, Beuker 1994). The eastern and more continental provenances generally have a late growth start, and the latest flushing populations come from Byelorussia, north-eastern Poland and the interior of the Baltic Republics. The variation in bud flushing and initiation of shoot growth of Norway spruce provenances are assumed to be regulated both by differential responses to accumulated temperature sums in the spring and by conditions during acclimation the preceeding year (Heide 1974b, Schmidt-Vogt 1977, Dormling 1982).

Photoperiod is the environmental factor that initiates the cessation of growth and development of frost hardiness (Dormling 1973), but with some modifications caused by temperature (Heide 1974a). Under controlled growing conditions in growth chambers, seedlings of provenances from the northernmost latitudes will respond with a terminal bud set at a night length of two to three hours, compared to eight to nine hours of darkness for south-western European origins (Dormling 1973). The northern Scandinavian and Finnish provenances, and those from high altitudes in the Alps, have the earliest cessation of shoot growth (Skroppa and Magnussen 1993). The latest growth cessation occurs in provenances from southern Poland and the eastern Carpathians.

At the provenance level, strong relationships are generally present between traits that characterise the timing and duration of the growth period, the lignification of the annual ring, and the development of autumn frost hardness (Skroppa and Magnussen 1993, Ekberg *et al.* 1994). These traits are components of an annual sequence of developmental events which describe the inherent annual rhythm of trees of the same provenance (Sarvas 1972, Skroppa and Magnussen 1993).

Resistance to late spring frost is closely related to the time of growth start; the late flushing provenances from eastern Europe suffer less damage than native Nordic provenances during spring frost events in Scandinavia (Werner *et al.* 1991). A similar but somewhat weaker relationship is present between the timing of growth cessation and resistance to autumn frost. Provenances with early termination growth will normally enter dormancy (develop frost hardness) earlier than those extending their growth period late in the summer, and thus be less damaged by early autumn frosts. In the Alps, high altitude provenances will be more resistant to early autumn frosts than those of lowland origins (Holzer 1993).

The growth capacity of different provenances is closely related to the duration of their growth period (Holzer 1993, Skroppa and Magnussen 1993). Northern provenances, or those from high altitudes that are adapted to a short growth season, will therefore have a poorer growth potential than those adapted to a longer season. Two provenance regions with particularly high growth potential have been identified (Schmidt-Vogt 1978). One covers parts of the post-glacial advance of spruce from the Russian refugium, including the Baltic Republics, north-eastern Poland and northern parts of Byelorussia. The other region covers the eastern Carpathian and Bihor Mountains and parts of the Beskids.

Wood quality traits, such as basic wood density and its determining components, have been shown to vary considerably among different provenances (Mergen *et al.* 1964, Worrall 1970, Schmidt-Vogt 1986). They are also influenced by the extent of provenance transfer. Strong relationships exist between these traits and annual growth rhythm characteristics.

Provenance variation has been shown for a number of other traits, such as nutrient demands, respiration activity and shade tolerance (Schmidt-Vogt 1977). Provenance differences are present in crown form, related to snow and ice break resistance, with the resistance increasing according to altitude (Holzer 1964, Schmidt-Vogt 1977).

Enzyme genetic marker studies reveal a great genetic variability within Norway spruce populations (Müller-Starck *et al.* 1992, Goncharenko *et al.* 1995), and also in populations close to the climatic margin of the species (Tigerstedt 1973, 1979). In the most comprehensive isozyme study on Norway spruce (Lagercrantz and Ryman 1990), only 5% of the total genetic diversity was explained by differences among provenances. Some differentiation occurs among populations derived from different glacial refugia and appear to reflect their post-glacial evolutionary history (Lagercrantz and Ryman 1990). Populations from the same region show little genetic differentiation (Bergmann 1973, Lundkvist and Rudin 1977, Lundkvist 1979, Konnert and Franke 1991). Central European provenances appear to have reduced genetic diversity, expressed by a reduced level of average heterozygosity compared to those from eastern Europe and Scandinavia (Lagercrantz and Ryman 1990, Goncharenko *et al.* 1995).

Patterns of provenance variation show geographic variability on a large scale. However, genetic variation may be present between offspring from populations within the same provenance, and this variation is often larger than that between provenances. Dietrichson (1973) sampled three populations from the same altitude (620-750 m) in each of five provenances, covering an area of 200 km north-south and 250 km west-east in southern Norway. Measurements were made of heights at two and four years, growth initiation and cessation, and lignification of the annual ring. Several traits showed a larger variation among populations within a provenance than among provenances. In studies of clones sampled from several populations from the same provenance region and from different provenances, variation among clones has

been demonstrated for a large number of traits (Sauer *et al.* 1973, Sauer-Stegmann *et al.* 1978, Kleinschmit *et al.* 1981, 1981).

### B. Variability within populations

The large within-population genetic variation demonstrated by genetic markers has been confirmed in studies of quantitative traits. Genetic variability has been found within all natural Norway spruce populations studied, as well as for traits that show clinal variation at the provenance level (Dietrichson 1971, 1973, Eriksson 1982, Skråppa 1982a, Ekberg *et al.* 1985, 1991). Traits that show such variation characterise germination, early and later height and diameter growth, the timing and duration of the shoot growth period, autumn frost hardiness, survival in the field, and branch, crown form, stem and wood quality (Schmidt-Vogt 1977, Skråppa 1991, 1993, Hylen 1997). The range of variation may sometimes be as large as that found between geographically distant provenances.

### C. Resistance to fungi and insects

Studies investigating Norway spruce's resistance to root rot (*Heterobasidion annosum*) have been carried out at both the provenance and clonal level (Dimitri and Kliefoth 1980). Treschow (1958) found no variation in growth of *H. annosum* among trees of different provenances. In an inoculation experiment with *H. annosum* on 98 Norway spruce clones, differences among clones were found in lesion length and fungal growth in sapwood (Swedjemark and Stenlid 1996). This indicates the presence of genetic variation among individual clones in degree of resistance to the fungus, and indicates that progress in resistance can be achieved through selection. Similar variation among clones has been found in resistance to bark beetle fungus infection (*Ceratocystis polonica*) (Christiansen and Berryman 1995, Brignolas *et al.* 1995).

Differences have been demonstrated among provenances in respect to infestation by spruce aphids (*Adelges* spp.) (Balut and Sabor 1993), and also among families and clones from the same population (Skråppa, unpublished). Little information is available on genetic variation in resistance to attacks by other insect species.

### D. Factors influencing the genetic variability

The great genetic variability of the Norway spruce forests is influenced by a large number of factors: ancient origin and immigration history, natural selection, an extensive gene flow caused by pollen dispersal, genetic drift due to small population size, and human activities.

Different factors may cause specific variation patterns to be present in parts of the range of the species. As an example, in some areas the existence of frost pockets or different slopes and exposure aspects may have resulted in selection of different annual growth rhythms. Patterns of spacial differentiation may therefore be the result of complex interactions of gene flow and selection (Finkelday 1995, Krutovskii and Bergmann 1995). In central Europe the species has been cultivated for more than 300 years, partly with seed material transferred from other regions. Differences in performance between provenances from the same region therefore may not exclusively express adaptational differences. Recent experimental results indicate that phenotypic provenance variation in traits characterising climatic adaptation is not only regulated by classical (Mendelian) gene frequency differences, but also by other mechanisms (*e.g.* gene regulation). These mechanisms appear to be triggered by environmental influences during the generative reproductive process (Skråppa and Johnsen 1994, Johnsen and Skråppa 1996, Johnsen *et al.* 1995, 1996).

## 7. Ecology

### A. Synecology and associated species

Spruce forests are found in many different habitats. They usually belong to the acidophilous order *Vaccinio-Piceetalia* and to a lesser extent to the *Fagetalia* (Ellenberg 1988, Fremstad 1997).

Forests of *P. abies* play a dominating role in the boreal zone in Fennoscandia and northern Russia. They belong to three associations, according to Kielland-Lund (1981, 1994):

- *Eu-Piceetum* (EP). This is the most common forest association. EP is the typical climax community on nutrient poor to medium rich, podzolic soil types. EP is subdivided into sub-associations, e.g. *myrtilletosum* (= “spruce forest with bilberry”), *dryopteridetosum* (= “spruce forest with small ferns”) and *athyrietosum* (= “spruce forest with tall ferns”), with increasing soil richness and soil humidity (Dahl *et al.* 1986, Kielland-Lund 1994).
- *Melico-Piceetum* (MP). According to Kielland-Lund (1994), MP (= “spruce forest with low herbs”) occurs in warmer localities and on more calcareous soils than *Eu-Piceetum*, mainly in the boreonemoral and south boreal zones. MP has three main sub-associations: *pinetosum* (= “calcareous low-herb woodland”) on dry limestone soils, *typicum* (= “spruce forest with low herbs”) and *aconitetosum* (= “spruce forest with tall herbs”). Most *P. abies* forests have a trivial vascular plant flora, except *Melico-Piceetum pinetosum*, which houses, among others, rare and in some areas threatened orchids, e.g. *Ophrys insectifera* and *Cypripedium calceolus*.
- Kielland-Lund (1981, 1994) has described the association *Chamaemoro-Piceetum* on clay or thin organic soils. This community is called “spruce swamp forest”.

Norway spruce forest communities in the superhumid parts of central Norway differ from those described (Kielland-Lund 1981, 1994) in having frequent oceanic species: in the field layer, e.g. *Blechnum spicant* and *Cornus suecica*; and in the bottom layer, the sub-oceanic bryophytes *Plagiothecium undulatum* and *Rhytidiadelphus loreus*.

The most common types of central European spruce forest are the montane and the sub-alpine. Spruce also occurs in wide areas in lowlands with mixed woodland communities. It can be dominant at both the sub-montane and planar levels where there is low competition, e.g. around the edges of raised bogs, in acid marshy ground and on waterlogged soils. Four montane and sub-alpine *Vaccinio-Piceetalia* associations are described:

- *Piceetum montanum* (PM). According to Ellenberg (1988), PM occurs in the montane zone of the valleys of the Alps. PM has two main sub-associations: *galietosum* and *melicetosum* (= *Melico-Piceetum*). The slightly humid *galietosum* is found where the substrate is rich in bases. It has many herbs, predominantly *Galium rotundifolium*. The dry montane *melicetosum* is poorer in species. This type also occurs on bedrock, which is poor in bases, and even on dry slopes with relatively base-rich and loamy soils.
- *Veronico urticifoliae-Piceetum* (VP). VP is a special association of a more productive montane spruce wood type (Ellenberg 1988). It develops on acid soil where the water supply is somewhat better than that where *Piceetum montanum melicetosum* woods are found.
- *Piceetum subalpinum* (PS). In contrast to the montane spruce woods, the sub-alpine woods are as a rule poorer in species (Ellenberg 1988). These are more constant and may appear in large

numbers, e.g. *Oxalis acetosella*, *Vaccinium myrtillus*, *V. vitis-idaea*, *Calamagrostis villosa* and *Hylocomium splendens*. The spruce trees are mostly stunted because of severe winters. The type is common in central European highlands.

- *Sphagno-Piceetum* (SP) or *Piceetum subalpinum sphagnetosum*. This type, which has plants associated with wet conditions such as the genus *Sphagnum*, is found on waterlogged soils in all acidophilous spruce woods, especially in the sub-alpine region. It is frequent in high precipitation areas along the perimeter of the Alps.

The symbiotic relationship between the roots of Norway spruce and mycorrhiza fungi is important for spruce forest ecosystems. The importance specifically concerns Norway spruce in dry habitats, in habitats where soil moisture is variable, or generally in habitats with marginal growing conditions. In optimal growing conditions for Norway spruce, mycorrhizae are not so well developed. In dry habitats, mycorrhizae facilitate water uptake. Hundreds of species of mycorrhizae are described on Norway spruce.

### B. Norway spruce as a key species

Norway spruce's importance for a very large number of species is due to its ability to change the soil, and to create essential structures owing to the size of individuals and their distribution in large continuous forests, as well as its dominance in creating dynamics in the landscape. In addition, Norway spruce as growing tree and decaying wood provides "home and food" for hundreds of species. In Fennoscandia and northern Russia, it dominates the forest landscape as the region's key species. In Norway, an estimated 20 000 species (~ half the number of species in the country) are associated with forests, a major part to spruce forests. Almost half the threatened species in Norway, Sweden and Finland (898, 695 and 717, respectively) live in forests (data from Nord 1994).

### C. Special lichens

The spruce forest (called "boreal rain forest" or "coastal rain forest") of the west central part of Norway is very rich in mosses, fungi and lichens. The latter group includes 40-50 species belonging to the so-called "Trøndelag element". The lichen species either have their only known occurrence in Europe in this forest type, or have their main occurrence here (Holien 1996). A good indicator group for the occurrence of rare epiphytic lichens in the coastal rain forest is the *Lobarion* community, for which *Lobarion pulmonaria* is a characteristic species. In the alliance *Lobarion*, the epiphytes *Pseudocyphellaria crocata* and *Ramalina thrausta* are considered among the vulnerable Red List species of the coastal rain forest, whereas *Pannaria ahlneri* and "trønderlav" (*Erioderma pedicellatum*) are highly endangered. "Trønderlav" was classified as extinct in Europe until the summer of 1994, when it was found in two extremely small populations.

### D. Special bryophytes

Norway spruce forests have a wide range of microclimatic and edaphic niches for bryophytes. Logs of Norway spruce at various stages of decay are habitats for many more or less specialised and very often rare communities and species of liverwort. Liverwort's occurrence on Norway spruce logs depends mainly on two factors: (i) the stage of decay (or length of time since the tree was felled) and (ii) the size of the log. Different species have different preferences. Larger logs have more species and larger populations. Typical Red List species living on decaying logs are *Lophozia ascendens* and *Calypogeia suecica*. The former is a vulnerable species with a preference for large young logs, while the latter prefers large old (heavily decayed) ones. Both occur mainly in the superhumid spruce forest of central Norway.

### **E. Interaction between planted Norway spruce forests and other forest types**

The general consequences of artificial introduction of *P. abies* into deciduous forests are well known, especially from western Norway and central Europe (Ellenberg 1988, Fylkesmannen i Rogaland 1993). The high shade tolerance of *P. abies* gives it a competitive advantage over nearly all deciduous species, and over ground flora if the temperature regimes are favourable for Norway spruce. The microclimate becomes more humid and oceanic after introduction of *P. abies*. In addition, the humus becomes rawer and more acid. Changes in microclimatic and edaphic conditions result in sparse ground flora and fauna (Børset 1985). Under natural conditions there is a balanced dynamic in the competition between *P. abies* and trees and communities. However, when spruce stands are introduced outside their natural range of occurrence, the species can show unpredictable invasiveness.

### **F. Elements of boreal spruce forest ecosystems**

Due to Norway spruce's dominant role in Fennoscandia and northern Russia, there should be a strong focus on the significance of the structure and dynamics of boreal spruce forests (Hansson 1992), especially in these areas.

### **G. Tree species**

Different tree species are normally dominant at different succession stages. There are exceptions, however, in a few vegetation types in the boreal zone where the same tree species is both the pioneer and climax species. Spruce forests go through a deciduous stage (mainly birch, rowen, aspen, *Salix* sp. and alder in Fennoscandia) as the first step before the spruce becomes dominant. The biodiversity of the spruce forest depends on its succession stage (reviewed in Tømmerås 1994).

### **H. Deciduous trees in conifer forests**

Deciduous trees lose their dominance in spruce forests after the pioneer period, but some of these trees are always part of the forest stand. The richer the vegetation type, the more deciduous trees are present. Old deciduous trees and large dead ones are very important for numerous lichens, insects and birds.

### **I. Period of rotation**

Plant and animal species adapt to many niches, in a complicated pattern, during the forest's successive stages. Species that require very specific conditions may be dependant on the presence of burned trees, dead wood, small seedlings, or old but living trees. The effects of disturbances in the rotation cycle on species diversity are not well known (Hansson 1992).

### **J. Layers**

From an ecological point of view, the existence of more than one vertical layer in forests is a key factor in determining an area's biodiversity. This layer structure is most dominant late in the pioneer and later stages.

### **K. Old trees**

Spruce in natural forests may reach an age of 200-300 years. Usually some individuals become old and are of great size. These trees are habitats for many forms of life, such as woodpeckers, lichens, bryophytes and insects.

#### **L. Dead trees**

Dead wood results from various causes, including storm felling, fire, pathogens and normal ageing. This leads to a wide spectrum of types of dead wood being found in a spruce forest. Over a thousand plant and animal species take part in the process of decomposing dead spruce; a large proportion are specialists at a particular stage. It is estimated that more than 700 Norwegian beetle species are dependent on the dead wood of boreal forest tree species (Tømmerås, unpubl).

#### **M. Continuity**

Some areas in boreal forests are free from natural disturbances such as fire and storm felling. These forest areas can cover 20-40% of a forest landscape, and are often widespread on humid soil and in steep valley areas. Many cryptogams and invertebrates are dependent on continuous forest structure.

#### **N. Importance of structural conditions and dynamic processes for plants and animals**

The natural dynamics of spruce forests, in which a special mosaic landscape changes over time, leave some parts very little affected by disturbances (continuous forest) and other parts dominated by, for example, fires once every 100 years. Plant and animal communities adapt to these conditions. Many species are dependent on the stable structural conditions of a continuous forest, while others need disturbances such as fire (*e.g.* the threatened ortolan bunting *Emberiza hortulane* L., many insects and fungi) or storm felling. Finally, many species are dependent on the mosaic combination at the landscape level. Among these are the three species of forest hens: black grouse (*Tetrao tetrix* L.), willow grouse (*Lagopus lagopus* L.) and hazel grouse (*Bonasa bonasia* L.).

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## SECTION 4 POPLAR (*POPULUS* L.)

### 1. Forestry Practices

It has been estimated that more than 90% of poplar cultivation throughout the world is concentrated on species and hybrids in section *Aigeiros* (Thielges 1985). This is due to the ease of intrasectional hybridisation and intersectional crosses with species in the *Tacamahaca* section, the broad adaptability of these species and hybrids in the temperate and sub-tropical zones, and the ease of vegetative propagation. Section *Turanga* has been increasing in importance, and the scale of some planting programs is enormous: the Three North Shelterbelt System is a 35.6 million hectare shelterbelt project across the desert border in northern China, where poplars make up 60% of the effort, much of it *P. euphratica* and hybrids *Populus simonii* × *P. nigra* (Weisberger *et al.* 1995, Wang 1996).

#### A. Deployment of reforestation materials

Seedling propagation of reforestation stock is most commonly used for difficult-to-root species in section *Populus*, although there are no significant reforestation efforts with species in this section in Canada (PCC 1996b). When practised, primarily for growing out controlled crosses, seed propagation is easily carried out using standard greenhouse propagation techniques (Burr 1986, Stanton and Villar 1996).

For the most part, stem cuttings from species in the *Tacamahaca* and *Aigeiros* sections root easily, while rooting is usually poor for those in sections *Populus*, *Leucoides* and *Turanga* (Zsuffa 1975). The rooting ability of *P. deltoides* varies, while that of *P. nigra* and *P. balsamifera* is very high. These latter species transmit their better rooting ability to hybrids with *P. deltoides* (Zsuffa *et al.* 1993). Vegetative propagation techniques for poplars in nursery culture can be divided into two groupings: autovegetative propagation, including cuttings, layering, etc., and heterovegetative propagation, including grafting, budding, etc. (Fröhlich and van der Meiden 1979).

For easy-to-root varieties, stem cuttings are normally taken from ripened one-year-old shoots during the dormant season, usually from plants in nursery stool beds, but sometimes from epicormic branches of older trees. Such varieties are often planted directly as unrooted cuttings or as pre-rooted stock on well-prepared planting sites. For harder-to-root varieties, more intensive rooting procedures must normally be used, often using greenwood material, rooting hormones and mist chamber techniques. Poplars from section *Populus*, that are notoriously difficult to propagate from stem cuttings, are more commonly propagated from root suckers, root cuttings and layers (Benson and Schwalbach 1970, Zsuffa 1971, Dirr and Heuser 1987, Hall *et al.* 1989).

Grafting and budding of difficult-to-root varieties is possible, and some species combinations demonstrate that intersectional compatibility is present, for example *P. tremula* on *P. trichocarpa* (Dirr and Heuser 1987), and *P. alba* or *P. × canescens* on *P. lasiocarpa* (Fröhlich and van der Meiden 1979) is possible: the most extensive application of grafting as a propagation technique has likely been in China where *P. × tomentosa* (*P. alba* × *P. adenopoda*), a variety that roots poorly from stem cuttings, is grafted onto *P. simonii* or one of its hybrids. If the grafted plant is planted with the union below the soil surface, it is felt that the scion forms its own roots over time (Zsuffa *et al.* 1996).

Poplars are also amenable to propagation by tissue culture and various protocols and explant materials have been used. The difficult-to-root species from section *Populus* have been propagated *in vitro* by enhanced axillary branching of shoots. Other cultivars of *P. × canadensis* (*P. deltoides* × *P. nigra*) and *P. yunnanensis* are propagated *in vitro* using dormant buds as the explant source (Dirr and Heuser 1987). *In vitro* techniques offer the most likely means for vegetative propagation of species and hybrids in section *Populus* (Frühlich and Weisgerber 1985, Ahuja 1987). The use of somatic embryogenesis has proved successful with *P. alba* × *P. grandidentata* (Michler and Bauer 1991).

Embryo culture (also referred to as embryo rescue) techniques have been developed to improve the recovery rates of hybrid genotypes where crosses often exhibit high frequencies of aborted or immature embryos (Stanton and Villar 1996). While it has been possible to culture individual embryos or whole ovules removed several weeks after pollination (Kouider *et al.* 1984, Savka *et al.* 1987), subsequent developments have allowed the culture of half capsules or individual carpels, followed by subculture of germinated embryos (Raquin *et al.* 1993).

Poplars are deployed not only in production plantations, but are also important species for protection planting, especially as windbreaks on the plains for North America, and for other landscape use. Plantations may be deployed as monoclonal stands, mosaics of monoclonal blocks or clonal rows, and as intimate single-tree mixtures of various genotypes (Zsuffa 1993).

While Canada presently has no regulations governing the control and certification of planting stock, a certification service has been designed and introduced by the Poplar Council of Canada. The service provides certification of: (1) varietal (clonal) identity; (2) quality and type; and (3) sanitary condition. In addition the service maintains a Canadian register of clones and varieties *not* recommended for planting (Zsuffa 1993, PCC 1996a). In Europe, the 130 cultivars registered for commercial use in any country of the European Union (EU) can be marketed within the EU since 1966, and their circulation became free in 1993 with the opening of the single market (Pinon and Valadon, 1997). In Germany, *Populus* reproductive materials are regulated by the Gesetz über forstliches Saat-und Pflanzgut (FSaatG).

## B. Provenance transfer

Although large geographic variation has been observed among provenances in morphology, growth and wood properties, the results to date indicate that possibilities for gain “vary from encouraging to confusing to bleak” (Farmer 1996). Limited studies in various *Populus* species do suggest that productivity gains might be achieved through provenance transfer (*e.g.*, Nelson and Tauer 1987), but the focus on hybrid breeding and clonal selection has precluded serious consideration of provenance transfer as an improvement strategy.

## C. Breeding programs

Poplar breeding has been characterised by two main features: interspecific hybridisation and clonal selection (Bisoffi and Gullberg 1996). The spontaneous occurrence of natural hybrids among sympatric and introduced species was recognised early this century and suggested a logical starting point for breeding programs. The more often assumed rather than proven superiority of F1 hybrids has been based on performance of selected clones usually attributed to heterosis, although recent studies have confirmed the existence of hybrid vigor (Stettler *et al.* 1988, Bradshaw and Stettler 1995). The incorporation of clonal selection as a characteristic of poplar breeding programs is largely due to the relative ease of vegetative propagation, compared with sexual reproduction (Mohrdiek 1983, Thielges 1985).

Interest in the breeding of polyploids followed the discovery of fast-growing triploid aspens in the 1930s (Einspahr *et al.* 1963, Einspahr and Winton 1976) but largely declined in the 1970s, with the

exception of some molecular genetics studies (Bradshaw and Stettler 1993). Renewed interest in aspen breeding in western Canada and the Lake States has focused on hybrid vigour of crosses between *P. tremuloides* and *P. tremula*, followed by clonal selection and deployment (Li and Wyckoff 1991, Li *et al.* 1993, Li 1995).

Important long-term breeding programmes have a long history in Europe (Italy, France, Belgium, Netherlands). More recently, co-operation between universities and pulp industries has led to a very intensive effort in the Pacific Northwest region of North America concentrating on *P. trichocarpa* × *P. deltoides* hybrids, and to a lesser extent those of *P. trichocarpa* × *P. maximowiczii*, *P. trichocarpa* × *P. nigra*, and *P. deltoides* × *P. nigra* (Stettler *et al.* 1996b, Zsuffa *et al.* 1996). In Bavaria in Germany cross breeding between *P. maximowiczii* × *P. trichocarpa* and *P. maximowiczii* × *P. nigra* has also been conducted. Intensively managed, short-rotation (5-8 years) plantations are geared toward pulp production, and over 30,000 ha are under cultivation as part of this programme between southern Oregon and British Columbia (Zsuffa *et al.* 1996).

Since the modern techniques of molecular biology are now being successfully applied to *Populus* it can be anticipated that novel traits, such as herbicide resistance, insect resistance and modified wood characteristics, will be introduced into these species.

#### D. Conservation of genetic resources

The history of poplar breeding and intensive culture spans a 70-year period. The International Poplar Commission (IPC) was established in 1947 to assist in the direction and co-ordination of this effort and to promote conservation and exchange of germplasm in 35 member nations (IPC 1996). In 1992, the IPC formally requested its member countries “to adopt appropriate measures to ensure that existing genetic resources of poplar and willow species, in natural and man-made stands, be properly preserved, stressing the role that fast-growing species may play in reducing pressure on delicate and endangered natural environments world-wide” (IPC 1992).

Some species of *Populus* are near the point of disappearing in some parts of their natural range, for example, *P. nigra* in Western Europe, while other species are still in full evolution, for example, *P. deltoides*. IPC nations have thus been encouraged to develop strategies for *in situ* conservation. Such efforts started in North China with the *ex-situ* conservation of *Populus simonii* due to three FAO co-ordinated poplar projects (Weisgerber *et al.* 1995), and in Europe with the conservation of *P. nigra* by the EUFORGEN network (Turok *et al.* 1997, Cagelli and Lefèvre, 1996). For broadly distributed species like *P. deltoides*, the recommended focus of *in situ* conservation is on small, isolated populations at the limits of the species range, as a source of adaptive variation. Once widely distributed, much of the original genetic resource for *P. nigra* was lost when natural regeneration was excluded by human activity (Steenackers 1996). The disappearance of *P. nigra* from middle Europe may also have resulted from the backcrossing of hybrids, especially of *Populus* × *canadensis* and *P. nigra*.

Other poplar species have a restricted natural range and require special protection. *P. suaveolens* (syn. *P. maximowiczii*) is well protected in natural reserves at different altitudes in the mountains of Hokkaido and can freely regenerate from seed in these areas. *P. heterophylla* is another species with restricted distribution, growing on soils that are too wet for *P. deltoides*; a special strategy is required to protect this species *in situ* (Steenackers 1996).

Despite the long history of domestication, *ex situ* conservation efforts have been limited and rarely involve seed materials. A notable exception is the *P. trichocarpa* seed bank created by the Netherlands in the 1970s. Promotion of *ex situ* conservation efforts has been recommended as an imperative to the IPC, as

has the need for guidelines for the global management and conservation of poplar genetic resources (Steenackers 1996).

## 2. Taxonomy and Natural Distribution

Poplar species (*peuplier* in French) are members of the genus *Populus* L., in the family Salicaceae (willow family) and the order Salicales. The genus is traditionally subdivided taxonomically into sections. Five of these sections are widely recognised: *Turanga*, *Leucoides*, *Aigeiros*, *Tacamahaca*, and *Populus* (known synonymously as *Leuce*) (Zsuffa 1975). Periodically, taxonomists have been inclined to add a sixth single-species section to resolve classification problems. For example, Browicz (1966) proposed section *Tsavo*, to include the east African species *P. ilicifolia*, a species not even recognised by some taxonomists and included by others in section *Turanga*. Section *Ciliata* has been proposed to include the Himalayan species *P. ciliata* Wall. Ex Royle, formerly included in *Leucoides* (Khosla and Khurana 1982), an apparent mistake that others have suggested be resolved by classifying the species under *Tacamahaca*. Still another section, *Abaso*, has been proposed to accommodate *P. mexicana* that seems weakly related to other species of section *Aigeiros*, in which it has previously been placed (Eckenwalder 1996). The dispute over the sectional classification of poplars will no doubt continue; meanwhile, it is generally accepted that three of the sections are represented in Canada: *Populus*, *Aigeiros*, and *Tacamahaca* (Krüssmann 1985, Farrar 1995).

Disagreements over the species classification of poplars show no sign of abatement. The wide distribution of many poplar species, frequent introgressive hybridisation, a long history of cultivation and ease of vegetative propagation has led to much confusion in the nomenclature of poplars. Numerous synonyms exist, and hybrids and cultivated varieties have often been named as species (Zsuffa 1975). Thus, species counts for the genus range from the low 20's to over 80, depending on the authority. The classification suggested by Eckenwalder (1996), which enjoys the transitory advantage of being the most recently published, recognises 29. This classification is presented, with synonyms recognised by Zsuffa (1975), in Table 3.5. Polymorphisms of DNA add new data to this “conventional” classification (Cervera *et al.*, 1997).

### Section *Turanga* Bge

The three species in this section are native to northeast Africa and Asia. The most important is *P. euphratica* which, although not commonly cultivated in the past, can tolerate poor soils, extreme heat and soil salinity, and is now a key species for anti-desertification purposes in the large Three North Shelterbelt project in northern China (Wang 1996).

### Section *Leucoides* Spach – Large-leaved Poplars

While no members of this section are native to Canada, swamp cottonwood (*P. heterophylla*) is a secondary species on wet sites in the central and eastern United States. Other examples of this section, *P. lasiocarpa* and *P. glauca*, are native to temperate regions of China.

### Section *Tacamahaca* Spach – Balsam Poplars

North American members of this section, found in both Canada and the United States, are balsam poplar – *peuplier baumier* (*P. balsamifera*), black cottonwood – *peuplier de l'Ouest* (*P. trichocarpa*), and narrowleaf cottonwood – *peuplier à feuilles étroites* (*P. angustifolia*). This group includes the commonly planted Simon poplar – *peuplier de Simon* (*P. simonii*) from eastern Asia. Other important members from Asia include *P. laurifolia*, and *P. suaveolens*.

**Table 3.5 Suggested classification, nomenclature and occurrence of *Populus* species (Eckenwalder, 1996) and synonyms given by an earlier classification (Zsuffa, 1975) in square brackets**

Section	Scientific name & synonyms	Common names	Occurrence
<b>Abaso</b> Ecken.	<i>P. mexicana</i> Wesmael		Mexico
<b>Turanga</b> Bge.	<i>P. euphratica</i> Oliv. <i>P. ilicifolia</i> (Engler) Rouleau <i>P. pruinosa</i> Schrenk	Euphrates poplar, bahan	Spain, NE Africa, Asia E. Africa E. Eurasia
<b>Leucoides</b> Spach	<i>P. lasiocarpa</i> Oliv. <i>P. glauca</i> Haines [ <i>P. wilsonii</i> Schneid.]	large-leaved poplars Chinese necklace poplar	China China
	<i>P. heterophylla</i> L.	swamp cottonwood, swamp poplar,	USA
<b>Tacamahaca</b> Spach	<i>P. angustifolia</i> James	balsam poplars narrowleaf cottonwood, narrowleaf balsam poplar balsam poplar	southern Sask. And Alberta to southwestern US North America Himalayas eastern Asia eastern Asia NE China, Japan
	<i>P. balsamifera</i> L. <i>P. ciliata</i> Royle <i>P. laurifolia</i> Ledeb. <i>P. simonii</i> Carr. <i>P. suaveolens</i> Fish. [ <i>P. cathayana</i> Rehd. <i>P. koreana</i> Rehd <i>P. maximowiczii</i> A. Henry ] <i>P. szechuanica</i> Schneid. <i>P. trichocarpa</i> Torr. & A.Gray	laurel poplar Simon poplar doronoki, Japanese poplar	E. Eurasia western Canada and US
	<i>P. yunnanensis</i> Dode	black cottonwood, western balsam poplar	E. Eurasia
<b>Aigeiros</b> Duby	<i>P. deltoides</i> Marsh. [ <i>P. sargentii</i> Dode, <i>P. wislizenii</i> Sarg. ]	Cottonwoods and Black Poplars eastern cottonwood (ssp. <i>deltoides</i> ), plains cottonwood (ssp. <i>monilifera</i> ), Rio Grande cottonwood (ssp. <i>wislizenii</i> )	Quebec, Ontario Prairie Provinces to Texas SW USA
	<i>P. fremontii</i> S.Wats. <i>P. nigra</i> L.	Fremont cottonwood black poplar, European black poplar	SW USA Europe, western Asia
<b>Populus</b> L. [ <i>Leuce</i> Duby]	<i>P. adenopoda</i> Maxim. <i>P. alba</i> L.	aspens white poplar, silver poplar	central and southern Europe to N. Africa, central Asia E. Eurasia eastern North America
	<i>P. gamblei</i> Haines <i>P. grandidentata</i> Michx.	largetooth aspen, bigtooth aspen, aspen, poplar, popple	Mexico Mexico Japan Mexico
	<i>P. guzmanantensis</i> Vasq. & Cue. <i>P. monticola</i> Brand <i>P. sieboldii</i> Miq. <i>P. simaroa</i> Rzed. <i>P. tremula</i> L. [ <i>P. davidiana</i> (Dode) Schneid.] <i>P. tremuloides</i> Michx.	Siebold aspen, Japanese aspen European aspen, tremble, Zitterpappel trembling aspen, quaking aspen	Europe, northern Africa, north-eastern Asia North America

**Section Aigeiros Duby – Cottonwoods and Black Poplars.**

This section includes the “true” cottonwoods (a term also associated with *Tacamahaca*). In North America the section is represented by eastern cottonwood – *peuplier deltoide* (*P. deltoides* ssp. *deltoides*), and plains cottonwood – *peuplier deltoide de l'Ouest* (*P. deltoides* ssp. *monilifera*), found both in Canada and the United States, and by Fremont cottonwood (*P. fremontii*) and Rio Grande cottonwood (*P. deltoides* ssp. *wislizenii*) as secondary species in the southwestern United

States. The black poplar – *peuplier noir* (*P. nigra*) is an important species native to North Africa, central and western Europe, and the cultivar known as Lombardy poplar – *peuplier noir d'Italie* (*P. nigra* cv. 'Italica') is commonly planted as a hardy ornamental in North America.

### Section *Populus L. (syn. Leuce Duby) – Aspens.*

This section is further subdivided into two subsections, *Albidae* and *Trepidae*, containing the white poplars and aspens, respectively. The North American representatives of this section are both members of *Trepidae*: trembling aspen – *peuplier faux-tremble* (*P. tremuloides*), and largetooth aspen – *peuplier à grandes dents* (*P. grandidentata*). *P. tremula* is an important and highly variable aspen from Europe, while *P. sieboldii* is a recognised species from Japan. However, aspens throughout Eurasia are now thought to be races of a single, highly polymorphic species, viz. *P. tremula* (Barnes and Han 1993). While no white poplars are native to North America, the European white poplar – *peuplier blanc* (*P. alba*) was among the first species introduced from Europe.

Natural hybridisation has been reported between almost all sympatric poplar species, and between introduced and native poplars, both in North America and Europe (Schreiner 1974, Demeritt 1990). Natural hybridisation generally occurs between species in the same section to the limited extent that the parent species overlap (Brayshaw 1965, Eckenwalder 1977), although intersectional hybrids also occur. Species in different sections, though broadly sympatric, are ecologically isolated from one another, so that hybridisation occurs over large geographic areas but within a relatively narrow ecological range of overlap (Eckenwalder 1984a, c). Complicated natural hybrid populations may also form where three or more species are sympatric (Rood *et al.* 1986).

Eastern cottonwood was introduced into France from southeastern Canada in the late 1700's. In southern Germany, since the early 1970's, cross-breeding of *P. × canadensis* has been replaced by *P. trichocarpa × P. deltoides* or *P. trichocarpa × P.* Subsequent natural hybridisation with the native black poplar produced the hybrid which was named *P. × canadensis* in 1789 (Mühle Larsen 1960, Wright 1976). Clones of this hybrid are now widely planted across Europe. This hybrid was also the first poplar hybrid produced by controlled pollination, by Englishman A. Henry (Larsen 1956). Artificial hybridisation has been used in North America since the 1920s and 30s (Stout and Schreiner 1933, Heimbürger 1936). Several of the more important hybrids occurring naturally in North America are listed, together with synonyms and common names, in Table 3.6.

## 3. Centres of Origin/Diversity

### A. Natural distribution

The genus *Populus* is widely distributed throughout the Northern Hemisphere, in both the temperate and subtropical zones. Representative species are found from Alaska and Labrador south to northern Mexico, as well as Europe, North Africa, the Himalayas, mainland China and Japan (Schreiner 1974). Some species are very widely distributed. *P. tremuloides*, for example, is the most broadly distributed tree species in North America, spanning 110° of longitude and 47° of latitude, and the second most widely distributed in the world (Jones 1985, Barnes and Han 1993).

### B. Evolution and migrational history

It was long felt that *Populus* was one of the oldest contemporary angiosperm genus, originating in China and Japan during the Triassic; however, these fossil records are now associated with other taxa. While the closest relatives in Flacourtiaceae are from tropical Asia, the fossil record now indicates that the genus *Populus* had tropical origins in North America during the late Paleocene, about 58 million years ago

(Collinson 1992). These early leaf fossils are very similar to the present day *P. mexicana* in section *Abaso* (Eckenwalder 1996). In the late Eocene, the first Eurasian relatives from other sections appeared, with those of *Turanga* confined to the Old World, and an ancestor of section *Leucoides* invaded temperate habitats. During the Oligocene, precursors of *Tacamahaca* and *Aigeiros* appeared that would not become distinct sections until the Miocene, at which time members of section *Populus* also appeared (Collinson 1992, Eckenwalder 1996).

Table 3.6 Nomenclature of naturally occurring *Populus* Hybrids

Parentage	Hybrid designation	Common name
<i>P. alba</i> × <i>P. grandidentata</i>	<i>P. × roulwauiana</i> Boivin	
<i>P. alba</i> × <i>P. adenopoda</i>	<i>P. × tomentosa</i> Carr..	Chinese white poplar
<i>P. alba</i> × <i>P. tremula</i>	<i>P. × canescens</i> (Ait.) Sm.	grey poplar
<i>P. alba</i> × <i>P. tremuloides</i>	<i>P. × heimbürgeri</i> Boivin	
<i>P. angustifolia</i> × <i>P. deltoides</i>	<i>P. × acuminata</i> Rydb. [syn. <i>P. × andrewsii</i> Sarg.]	Lanceleaf cottonwood, <i>peuplier à feuilles acuminées</i>
<i>P. angustifolia</i> × <i>P. balsamifera</i>	<i>P. × brayshawii</i> Boivin	Brayshaw's poplar, <i>peuplier hybride de Brayshaw</i>
<i>P. angustifolia</i> × <i>P. tremuloides</i>	<i>P. × sennii</i> Boivin	
<i>P. balsamifera</i> × <i>P. deltoides</i>	<i>P. × jackii</i> Sarg.	Jack's poplar, <i>peuplier hybride de Jack</i>
<i>P. balsamifera</i> × <i>P. tremuloides</i>	<i>P. × dutillyi</i> Lepage	
<i>P. deltoides</i> × <i>P. nigra</i>	<i>P. × canadensis</i> Moench cv. Eugenei [syn. <i>P. × euramericana</i> (Dode) Guinier]	Carolina poplar, <i>peuplier de Caroline</i> [syn. Canada poplar, Euramerican poplars]
<i>P. deltoides</i> × <i>P. tremuloides</i>	<i>P. × bernardii</i> Boivin	Bernard poplars
<i>P. deltoides</i> × <i>P. trichocarpa</i>	<i>P. × generosa</i> Henry	Interamerican poplars
)	[syn. <i>P. × interamericana</i> Broekh.]	
<i>P. fremontii</i> × <i>P. trichocarpa</i>	<i>P. × parryi</i> Sarg.	Parry cottonwood
<i>P. grandidentata</i> × <i>P. tremuloides</i>	<i>P. × smithii</i> Boivin	
<i>P. laurifolia</i> × <i>P. nigra</i>	<i>P. × berolinensis</i> Dippel [syn. <i>P. × rasumowskyana</i> Schr. and <i>P. × petrowskyana</i> Schr.]	Berlin poplars, Russian poplars
<i>P. deltoides</i> × <i>P. balsamifera</i> × <i>P. angustifolia</i> (natural trihybrid)	Unnamed	Unnamed

The evolution of the advanced sections of *Populus* has been characterised by rapid speciation during allopatric cycles, but influenced by widespread introgression, both within and between sections (Eckenwalder 1984b, 1996, Smith and Symata 1990, Kaul 1995). This rapid sequence of events, much conflicting evidence, and the confusion that has characterised the identification of species, has made it difficult to track the recent evolutionary history of poplars in the more advanced sections (Eckenwalder 1996). While there is evidence of evolutionary divergence among the sections, the sections themselves are very widely distributed. Species within the sections are highly related and many are among the most broadly distributed of any tree species.

It is clear that migration of genes to other section members can occur easily throughout a very large portion of the North Temperate zone. Poplars are pioneering species and migrate quickly. Pollen studies have demonstrated that *Populus* species frequently dominate the first forest communities following glaciation (Cwynar 1988, Keenan and Cwynar 1992). In Europe, *P. tremula* is the early pioneering species. *P. nigra* occurs along rivers and in pastures, together with *Salix alba*. Large stands of *P. tremuloides* in North America are thought to have originated soon after retreat of the Pleistocene ice sheet and have been since maintained asexually from root suckers, making them some of the largest and oldest organisms in the world (Barnes 1975, Kemperman and Barnes 1976, Mitton and Grant 1980, Cheliak and Dancik 1982).

#### 4. Reproductive Biology

##### A. Reproductive development

Poplars are normally dioecious and obligatory outcrossers; however, the occurrence of monoecious inflorescences and perfect flowers has been reported (Lester 1963a, b, Melchior 1967). Reproductive buds in *P. tremuloides* may develop into pistillate, staminate or perfect flowers, initiated at different times (Lester 1963a). *P. lasiocarpa* is a notable exception as it is normally monoecious and self-fertilising (Schreiner 1974). Overall sex ratios of 1:1 have been confirmed for *P. tremuloides* (Einspahr and Winton 1976, Grant and Mitton 1979) and for *P. deltoides* (Farmer 1964b), although an elevational gradient in sex ratio has been observed in *P. tremuloides* in the Rocky Mountains, with females more common at low elevations, while more than 90% are male above 3 200 m (Grant and Mitton 1979).

Reproductive buds are simple (Jackson and Sweet 1972). Their initiation and early development have been described in *P. tremuloides* and *P. deltoides* (Nagaraj 1952, Seitz 1958, Lester 1963a). Floral initiation takes place in buds, located in the axils of leaves on the current-year shoot. These buds are no more than small apices, each with a single bud scale when winter dormancy occurs (Owens and Blake 1985, Kaul 1995). Terminal buds burst in May, and axillary primordia initiate several bud scales during rapid shoot elongation. Reproductive apices are determined around mid-June (Lester 1963a). Pistillate primordia are first to begin development of floral parts by late June, followed by staminate primordia in early July. In *P. deltoides*, staminate flower buds can be readily distinguished from vegetative buds by midsummer, while identification of pistillate flowers requires dissection (Farmer 1976). Floral development continues within buds through September, so that anthers and ovules are well developed before winter dormancy (Owens and Blake 1985, Kaul 1995). For at least some *Populus* species, a chilling requirement must be satisfied before development will resume (Farmer 1964a). Megaspore mother cells differentiate in the spring, and microsporogenesis immediately precedes anthesis (Farmer and Pitcher 1981).

Flowers are borne in catkins (aments) early in the spring, prior to flushing of vegetative buds. When fully developed, both male and female catkins are 10 to 15 cm in length. Female flowers have from two to four, cap- or y-shaped stigmas, while the males have 30 to 80 stamens (Demeritt 1990). Each catkin bears a few dozen, one-celled capsules, each containing 10 to 30 seeds.

Male flowers ripen and shed pollen a few days before females, ensuring that pollen is in the air when the first females are receptive (Farmer and Pitcher 1981). Such pollen-pistil interactions have been largely documented in *Populus nigra* (Villar *et al.* 1987a, Villar *et al.* 1993). Variation in flowering date is due to differences among trees and in *P. deltoides* is highly heritable (Farmer 1976). This variation in flowering date extends the pollination period from 2 to 3 weeks. Another study of natural variation in *P. nigra* over 111 sites in France revealed a fair level of diversity and a low overall differentiation, with an important intraregional gene diversity (Legionnet *et al.* 1997).

Pollen germinates within the first few hours after pollination. Fertilisation takes place several days later and is normally complete within two weeks (Farmer and Pitcher 1981). Seed development proceeds rapidly and dispersal occurs in most species by midsummer, before the full growth of the leaves (Schreiner 1974). In North American, the period of seed maturity in sections *Populus* and *Tacamahaca* is determined by temperature sums and is quite uniform within the limits of ecotypic zones (Pauley 1950). On the other hand, seed dispersal in the *Aigeiros* poplars may continue throughout the summer and early fall (Farmer 1966).

## B. Mating system and gene flow

Two factors contribute to high gene flow and genetic diversity in poplars. Firstly, most are dioecious, and thus obligatory outcrossers. And secondly, in addition to being wind-pollinated, the long white, silky hairs attached to the short stalks of the seeds promote wind dispersal over great distances (Schreiner 1974), resulting in high rates of migration.

Electrophoretic studies in *P. tremuloides* suggest that gene flow is high, leading to a lack of differentiation among populations for putative neutral allozyme loci. However, the role of non-random mating in these same studies was variable, with no deviations from Hardy-Weinberg equilibrium detected in populations sampled in Minnesota (Lund *et al.* 1992), while an excess of heterozygotes were found in Alberta populations (Cheliak and Dancik 1982), and a deficiency of heterozygotes observed in Ontario populations (Hyun *et al.* 1987).

## C. Seed production

Most poplars begin flowering between age 10 and 15 years (Schreiner 1974), although flowering in *P. deltoides* may occur as early as age four (Farmer and Pitcher 1981). *Tacamahaca* and *Aigeiros* poplars produce large annual seed crops. Those in section *Populus* produce some seeds each year, but bumper crops occur at intervals of three to five years. Poplars are prolific seed producers. A typical 12 m *P. deltoides* specimen was estimated to produce almost 28 million seeds in one season, and estimates for *P. tremula* have ranged as high as 54 million seeds. Poplar seeds are very small. Species in section *Populus* can produce 6000 to 8000 seeds per gram, while North American *Leucoides* and *Aigeiros* produce from 300 to 450 seeds per gram (Schreiner 1974).

Typically, the longevity of poplar seeds under natural conditions is quite short – about two to four weeks. Under controlled low-temperature (-18 to 5° C) and stable moisture content (5 to 8%) conditions, storage time has been extended to 140 days for *P. balsamifera* (Hellum 1973), two years for *P. tremuloides* (Fechner *et al.* 1981), and five to six years for *Aigeiros* poplars (Tauer 1979, Muller and Tessier du Cros 1982).

## D. Natural regeneration

Poplar seeds germinate or die within a few days after seedfall. Germination is epigeal. A fringe of hairs develops at the base of the hypocotyl, rendering the seedling upright and encouraging the root to grow down into the soil.

A favourable medium such as fine mineral soil is required for germination, together with light and continual moisture (McDonough 1979, Farrar 1995). Such conditions are rare, requiring fresh exposure of mineral soils, as found on shorelines, sandbars and old gravel pits. In North America, regeneration of section *Populus* from seed is confined to newly disturbed areas, whereas the primary mode of reproduction within stands is asexual (Barnes 1966, Schier 1973, Einspahr and Winton 1976).

## E. Vegetative reproduction in nature

Except for members of section *Populus*, all poplars sprout vigorously from the stump and root collar. Coppicing occurs occasionally on young aspen (Zsuffa 1975). Reproduction from adventitious shoots on roots (root suckers) is common in many species, although less frequent in those in the *Aigeiros* and *Leucoides* sections.

Clonal groups of *P. tremuloides* in eastern North America are very common, but generally less than 0.1 ha in size, while in areas of Utah, groups as large as 80 ha have been observed (Kemperman and

Barnes 1976). In the semi-arid western United States, some argue that widespread seedling establishment has not occurred since the last glaciation, some 10,000 years ago (Einspahr and Winton 1976, McDonough 1985). Indeed, some biologists feel that western clones could be as old as 1 million years (Barnes 1966, 1975). It has been claimed that a single clone, nicknamed "Pando" (Latin for *I spread*), covers 43 hectares, contains more than 47,000 stems and weighs in excess of 6 million kg, making it the largest known organism (Grant *et al.* 1992, Mitton and Grant 1996).

Studies have also demonstrated that both natural and vegetative propagation occur in nature, for example with *P. nigra* (Legionnet *et al.* 1997).

## 5. Genetics

### A. Cytology

Poplars are normally found in the diploid condition with  $2n = 38$  chromosomes (Blackburn and Harrison 1924, Smith 1943). Polyploid individuals are rare and have only been reported in a half-dozen species (Darlington and Wylie 1956). While rare, the first discovery of a triploid forest tree was, in fact, a clone of *P. tremula* (Müntzing 1936). Several other natural triploid clones have since been found in both *P. tremula* and *P. tremuloides*, usually exhibiting larger leaves and exceptional growth (Einspahr *et al.* 1963, Heimbürger 1968, Einspahr and Winton 1976).

Some reports suggest the sex determination in poplars is controlled by sex chromosomes (Peto 1938, Smith 1943, van Buijtenen and Einspahr 1959), however, this theory remains controversial. While published reports favour a genetic basis for gender, a linkage analysis of almost 2 500 PCR-based RAPD markers in a segregating family of F1 hybrid *P. trichocarpa* × *P. deltoides* failed to find any markers that were significantly associated with gender (McLetchie *et al.* 1994). The authors suggested that gender might be determined genetically by regions of the genome not sampled by the tested markers or by a complex of loci operating in an additive threshold manner or in an epistatic manner, or that gender is determined environmentally at an early zygote state.

### B. Genetic variation

As already mentioned, the genus *Populus* is tremendously varied with species distributed throughout the Northern Hemisphere and the opportunities to generate novel genotypes through hybridisation are enormous. Breeding programs have not hesitated to exploit this genetic variability, although sound quantitative estimates of narrow-sense and broad-sense heritabilities, and covariances among selection criteria would undoubtedly have assisted in making breeding and selection strategies more efficient (Riemenschneider *et al.* 1996). Poplars are ideal species for quantitative genetics studies, as clonal replication can be readily accomplished to describe complex modes of gene action (Foster and Shaw 1988, Mullin and Park 1992, Bradshaw and Foster 1992). It is thus surprising that genetic variation has been studied in detail for only a few species and traits.

The true potential of poplar species can only be determined by genetic studies designed to resolve variation among and within stands. Unfortunately, the concentration of breeders on interspecific hybridisation has left this field largely ignored, and detailed studies of large natural populations are quite recent and only a few species are well documented (Mohrdrick 1983, Farmer 1991).

#### *Population-level variability*

Considerable clonal variation among populations may be expressed for growth traits and for *Melampsora* rust resistance, but for other characteristics there is often little geographic differentiation. Overall, data from molecular genetic studies suggest that gene flow through migration has been sufficient

to prevent genetic drift, inbreeding, and other processes that might give rise to geographic variation unrelated to adaptive selection (Farmer 1996).

Significant variation over a 10° latitudinal transect was observed for phenology (Farmer *et al.* 1988a), shoot/root allometric coefficients (Schnekenburger and Farmer 1989), and height growth among four provenances of *P. balsamifera*, with southern sources continuing to grow later in the season (Schnekenburger and Farmer 1989, Farmer 1993). These same populations exhibited very little geographic differentiation for isozyme characters (Farmer *et al.* 1988a), rooting ability (Farmer *et al.* 1989), and date of bud break (Farmer and Reinholt 1986). Another test series including a more restricted sampling of populations over a 3.5° range found significant population differences accounting for about 12% of the variation in two-year height, leaf morphology, sylleptic branching and pest resistance (Riemenschneider *et al.* 1992), with populations grouped into north-western, central, and south-eastern clusters (Riemenschneider and McMahon 1993).

Similarly, in *P. tremuloides*, isozyme and RAPD variation studies have shown little differentiation among populations (e.g., Hyun *et al.* 1987, Lund *et al.* 1992, Yeh *et al.* 1995), whereas considerable variation among populations is well-documented for morphology, growth and wood properties (van Buijtenen *et al.* 1959, Barnes 1969, Einspahr and Winton 1976). Variation among populations generally follows clinal trends, with wood density declining with increasing elevation (Valentine 1962) and from south to north (Einspahr and Benson 1967). A common-garden trial showed that northern and western provenances flushed and ceased growth first, with lower survival when grown in Michigan (Brissette and Barnes 1984), and another showed that better growing clones came from lower Michigan (Reighard and Hanover 1985). There is some evidence of a north-south increase in susceptibility of *P. tremuloides* populations to *Hypoxylon mammatum* (French and Hart 1978). Populations of *P. tremuloides* also vary with respect to ozone sensitivity, with tolerance correlated to maximum daily ozone levels, as well as annual precipitation and minimum temperature (Berrang *et al.* 1991).

Geographic variation is also well documented for *P. deltoides*. A wide range provenance trial established in Nebraska, included sources from Texas in the south, to Minnesota in the north and Pennsylvania in the east, and evaluated bark, stem, crown and leaf morphology, in addition to growth and survival (Ying and Bagley 1976). Clinal patterns of variation from north and west to south and east were observed for most traits. Cuttings from Nebraska, Minnesota and Wisconsin produced significantly higher numbers of roots than those from other sources (Ying and Bagley 1977). A similar clinal trend was found in a study of 40 populations in the southern Great Plains, where NW to SE patterns were observed for two-year height, diameter, branching and *Melampsora* rust resistance (Nelson and Tauer 1987). A study of nine populations in Ontario showed great variation in leaf morphology which was unrelated to latitude or longitude, and was not correlated with the moderate allozyme variation that suggested differences between eastern and western populations (Rajora *et al.* 1991).

A series of studies of *P. trichocarpa* populations in Washington have documented significant variation for leaf, branch and phenology characters (Weber *et al.* 1985), photosynthetic processes (Dunlap *et al.* 1993), survival height growth and biomass production (Heilman and Stettler 1985), volume production, *Melampsora* rust resistance and adaptation to arid sites (Dunlap *et al.* 1994), leaf and crown morphology (Dunlap *et al.* 1995). A sample of 10 populations over a 4.5° latitudinal range showed only weak clinal trends for three-year height and diameter (Rogers *et al.* 1989). Another sample of five riparian populations in Washington showed little difference among populations with respect to flood tolerance of young seedlings and rooted cuttings (Smit 1988).

### Individual-level variability

While the amount of genetic variation among populations differs greatly depending on the trait, the variation within populations is moderate to high for virtually all traits. Unfortunately, most genetic testing has focused on clonal materials without any particular family structure, and these studies have concentrated on the species and hybrids of sections *Tacamahaca* and *Aigeiros*. Generally, only estimates of broad-sense heritability ( $H^2$ ) are available, and genetic structure is rarely partitioned into additive and non-additive components (Riemenschneider *et al.* 1996). In the limited number of studies of seedling populations, narrow-sense heritability ( $h^2$ ) estimates for growth were similar to estimates of  $H^2$  in *P. deltoides* (Farmer 1970, Ying and Bagley 1976, Nelson and Tauer 1987), and much lower in the case of *P. trichocarpa* (Rogers *et al.* 1989).

A large number of studies of growth and yield characters in *P. deltoides* have produced consistent estimates of  $H^2$  between 0.20 and 0.50, with significant genotype-environment interactions, usually less than half as large as the corresponding genetic main effects (Wilcox and Farmer 1967, Farmer and Wilcox 1968, Mohn and Randall 1971, 1973, Randall and Cooper 1973, Foster 1986). A limited number of clonally replicated trials suggest that much of the genetic variance in yield is non-additive (Foster 1985, Foster and Shaw 1988). Heritability for stem growth in *P. balsamifera* was about  $H^2 = 0.50$  (Farmer *et al.* 1988b). In both *P. deltoides* and *P. balsamifera*, C-effects (*in sensu* Lerner 1958) during the first year were often as large as that due to clones (Wilcox and Farmer 1968, Farmer *et al.* 1989), but appear to be less important after field planting (Farmer *et al.* 1988b). Detailed measurements of leaf, branch and phenological characteristics, which are known to affect tree productivity, have been employed to describe ideotypes (Dickmann and Keathley 1996) that may be useful in yield selection of *P. balsamifera* (Riemenschneider *et al.* 1992), *P. trichocarpa* (Riemenschneider *et al.* 1994), and hybrids involving *P. deltoides*, *P. nigra* and *P. simonii* (Wu 1994a, b). Indications of the utility of the ideotype concept for yield selection have been inconsistent.

Heritability of rooting and root characters is typically very high, with  $H^2$  estimates as high as 0.85 to 0.91 in *P. deltoides* (Wilcox and Farmer 1968, Ying and Bagley 1977). Other recent studies also indicate high heritability for rooting in *P. trichocarpa* (Riemenschneider *et al.* 1996).

Much effort has been concentrated on the inheritance of *Melampsora* rust resistance, due to its impact on poplar culture. Early studies of rust resistance in *P. deltoides* gave estimates of  $h^2$  between 0.38 and 0.66, and for  $H^2$  between 0.66 and 0.88 (Jokela 1966). Similarly high estimates for rust resistance or severity have since been reported for *P. deltoides* (Farmer and Wilcox 1968, Thielges and Adams 1975), *P. tremula* and *P. tremuloides* (Gallo *et al.* 1985), *P. balsamifera* (Riemenschneider *et al.* 1992), *P. trichocarpa* and its hybrids (Hsiang *et al.* 1993, Riemenschneider *et al.* 1994), and hybrids among *P. deltoides*, *P. nigra*, and *P. maximowiczii* (Rajora *et al.* 1994).

### Molecular genetics

In recent years a great deal of research has been directed towards associating important traits with molecular markers, and in developing corresponding genetic maps (Bradshaw *et al.* 1994, Cervera *et al.* 1997). The emphasis of this work has been directed towards adaptive traits (Bradshaw and Stettler, 1995) and to resistance to diseases (Villar *et al.* 1996), and suggests the role of a few quantitative trait loci (QTL's) that have large effects on these quantitative traits.

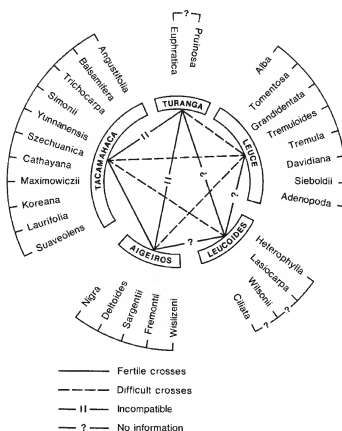
### C. Inbreeding depression and genetic load

Given the high rate of gene flow in poplars, one would expect low rates of inbreeding. A study of 200 clones of *P. tremuloides* in Ontario indicated a deficiency of heterozygotes and an average fixation index

of 0.462 (Hyun *et al.* 1987). However, these results might well be due to a sampling phenomenon, Wahlund's effect, rather than inbreeding. In contrast to these observations, populations of *P. tremuloides* in Alberta, where clonal reproduction is more common, showed an excess of heterozygotes (Cheliak and Dancik 1982, Jelinski and Cheliak 1992). While rates of inbreeding in natural populations may be low, genetic load may be expressed as inbreeding depression in pedigrees of hybrid material, giving rise to distortion of expected Mendelian segregation ratios (Bradshaw and Stettler 1994).

## 6. Crosses

Figure 3.4 Crossability of *Populus* species



Source : Zsuffa, 1975

Extensive crossability studies have been carried out among species in the *Populus*, *Tacamahaca* and *Aigeiros* sections, while few data are available for those in *Turanga* and *Leucoides* (Zsuffa 1975). Interspecific breeding results are summarised in Figure 3.4.

Hybrids between members of the same section are produced easily and are often more vigorous than their parents. Dramatic examples are the hybrids made between *P. tremuloides* and *P. tremula* (Ilstedt and Gullberg 1993). Crossing success between sections is variable. Crosses between *Aigeiros* and *Tacamahaca* are easy, while those between sections *Populus* and *Aigeiros*, and sections *Populus* and *Tacamahaca* are notoriously difficult, resulting normally in dead seed or dwarfed seedlings (Zsuffa 1975). Crosses between sections are sometimes made more easily using interspecific hybrids, rather than pure species, as parents.

The incompatibility of some species is characterised by arrested development of the pollen tube and failure to penetrate the stigma (Melchior and Seitz 1968, Guries and Stettler 1976, Stettler *et al.* 1980, Knox 1984, Villar *et al.* 1987b, Rougier *et al.* 1992, Villar *et al.* 1993). This block can be overcome in some cases by mixing the incompatible pollen with “mentor pollen”, *i.e.*, compatible pollen that has been killed by means of gamma radiation (Stettler and Ager 1984, Knox *et al.* 1987, Villar and Gaget-Faurobert 1996), and by treatment of pollen and stigmas with solvents and extracts from compatible pollen (Whitecross and Willing 1975, Willing and Pryor 1976). This technique has made it possible to obtain crosses of section *Populus* with *Aigeiros* and *Tacamahaca* poplars that are otherwise difficult (Stettler 1968, Zufa 1971, Knox *et al.* 1972, Willing and Pryor 1976).

## 7. Ecology and Associated Species

### A. Habitat

Poplars are found in a great variety of forest ecosystems, from boreal to sub-tropical, and from mountainous to riparian. In some environments such as boreal forests and in large river valleys, they form large stands, while in other situations they are found as small stands or groups of trees. Although there is some variation among species, essentially all poplars are very intolerant of shade. They are pioneering species and among the first to invade and re-colonise areas disturbed by harvesting, land clearing and fire. The *Populus* species and their hybrids vary greatly in their adaptability to climate, although all are nutrient demanding and perform best with an abundant and continuous supply of moisture (Heilman *et al.* 1996).

The members of section *Populus* are very broadly distributed and thus found over a tremendous range of climates. In *P. tremuloides*, the southern limit is roughly defined by the 24°C mean July temperature isotherm, while the northern limit corresponds to a mean annual degree-day sum of 700°C, at threshold temperature of 5.6°C (Fowells 1965). Within this range the species occurs where annual precipitation exceeds evapotranspiration. While *P. tremuloides* is found on a variety of soils, ranging from shallow and rocky to deep loamy sands and heavy clays, growth is strongly influenced by drainage and fertility (Perala 1990). Throughout the more restricted range of *P. grandidentata*, moisture is adequate in all seasons, with the least precipitation occurring at the north-west limit on the prairie border in Manitoba with only 510 mm. It is far less adaptable than *P. tremuloides*, developing best on moist, fertile sandy uplands with good aeration (Laidly 1990).

The members of the *Tacamahaca* and *Aigeiros* sections are referred to collectively as the riparian cottonwoods. Species in the *Tacamahaca* section are generally found at higher elevations and latitudes (high river systems of montane and young piedmont valley floodplains) (Braatne *et al.* 1996). In this section, *P. balsamifera* is the most broadly distributed, withstanding climatic extremes similar to that of *P. tremuloides*. It is usually restricted to moist, low-lying ground, and is one of the few boreal species associated with poorly drained clay soils having pH greater than 7.2 (Dix and Swan 1971, Zasada and Phipps 1990). *P. trichocarpa* is most commonly found in the humid coastal forests of the Pacific Northwest, performing best on deep alluvial soils with abundant moisture, nutrients, oxygen and pH 6.0 to 7.0 (Smith 1957, DeBell 1990). *P. angustifolia* has a very limited range where it is a pioneering species on gravel and sand bars near fast-flowing rivers (Brayshaw 1965).

Members of section *Aigeiros* are limited to lower elevations and latitudes (lower river systems of mature piedmont valley flood plains) (Braatne *et al.* 1996). The natural range of *P. deltoides* covers a wide southern range, with frost-free days ranging from less than 100 to more than 200, and rainfall from less than 380 mm in the northwest to more than 1,400 mm in the south. This species performs best on moist, well-drained, fine sandy or silt loams, and on sites that are rarely higher than 6 m above the average level of nearby streams (Cooper and Van Haverbeke 1990).

The only North American member of section *Leucoides*, *P. heterophylla*, is found in warm, humid areas with abundant rainfall. It performs best on deep moist soils of shallow swamps and low-lying areas near tidewater, and occupies sites that are too wet for *P. deltoides* (Johnson 1990).

## **B. Synecology and associated species**

Poplars occur in the early successional stages over a wide range of forest ecosystems, so it is no surprise that ecological associations are tremendously diverse. This is particularly true for species in section *Populus*, as they are not restricted to riparian habitats. In Europe, *P. tremula* is the early pioneer. *P. nigra* occurs along rivers and in pastures, together with *Salix alba*. *P. tremuloides* is found in pure stands across its range, but also in mixed stands where it is commonly associated with White Spruce (*Picea glauca*), black spruce (*Picea mariana*), balsam fir (*Abies balsamea*), white birch (*Betula papyrifera*), *P. balsamifera*, and jack pine (*Pinus banksiana*). The associations with shrub and herbaceous species are even more numerous and varied (Perala 1990, Farrar 1995). *P. grandidentata* occurs in small pure stands, but more commonly as an associate in poplar stands with either *P. tremuloides* or *P. balsamifera*. It is a minor component of many other forest types and is thus associated with a wide range of shrubs and ground flora (Laidly 1990, Farrar 1995).

In section *Tacamahaca*, *P. balsamifera* reaches its best development on river flood plains where it occurs as pure stands and is associated with various willows and alders (Viereck *et al.* 1983). However, it also occurs in mixtures with boreal conifers and several hardwood species, and the associated shrub and herbaceous mixtures are numerous (Zasada and Phipps 1990). *P. trichocarpa* generally occurs in mixtures with larger willows, and to a lesser extent with several western conifers (DeBell 1990). Shrub and herbaceous associates are numerous, but better sites are characterised by beaked hazel (*Corylus cornuta*), elder (*Sambucus* spp.), salmonberry (*Rubus spectabilis*), nettles (*Stachys* spp.), swordfern (*Polystichum munitum*) and lady fern (*Athyrium filix-femina*) (Smith 1957).

On riparian sites, *P. deltoides* tends to grow as essentially pure stands or mixed in open stands with other riparian species. In the area where the species performs best, roughleaf dogwood (*Cornus drummondii*) and swamp-privet (*Forestiera acuminata*) are major shrub associates (Cooper and Van Haverbeke 1990).

## **C. Competition and stand structure**

As mentioned earlier, all poplars are shade intolerant, early successional species, and disturbance is often essential in maintaining many poplar ecosystems. The availability of sites suitable for colonisation, particularly following fires, plays a major role in determining seedling establishment (DeByle and Winokur 1985, Jelinski and Cheliak 1992, Kay 1993). Once established, fire can also remove shade-tolerant competitors, allowing vigorous *P. tremuloides* sprouts to emerge from persistent root systems (Bailey *et al.* 1990), with densities in excess of 1 million per hectare (Schier *et al.* 1985). In the absence of disturbance, aspens are regarded as transient, and successional patterns are determined by soil water regime (Roberts and Richardson 1985). Intolerant associates will often out-live aspens, while tolerant hardwoods and conifers will also dominate by virtue of their ability to regenerate under shade.

The riparian cottonwoods are all very flood-tolerant, so that establishment and growth are promoted by the disturbances characteristic of alluvial habitats. In non-alluvial habitats, they take the opportunity to establish on moist agricultural fields, forest clearings and the margins of wetlands, but are eventually dominated by secondary forest species (Braatne *et al.* 1996). Willows and alders may precede the establishment of *P. balsamifera*, which is normally then replaced by White Spruce (Walker and Chapin 1986, Walker *et al.* 1986). *P. deltoides* is a very poor competitor, as it is very intolerant of shade, and

competes well only with willows, owing to its faster growth except on very wet sites (Cooper and Van Haverbeke 1990).

When established as plantations, poplars and their hybrids must generally be established in pure stands. In addition to being highly intolerant of shade, young poplars cannot tolerate competition from grass, weeds or shrubs. Control of vegetation in the first few years is essential, although poplars are highly sensitive to many herbicides used for vegetation control (Demeritt 1990).

#### D. Ecosystem dynamics

Poplars coexist with a wide range of insects, but most pose a more serious threat only to artificial populations of planted species and their hybrids. In North America the most serious defoliator, particularly in hybrid plantings, is the cottonwood leaf beetle (*Chrysomela scripta*). Other foliage insects include the forest tent caterpillar (*Malacosoma disstria*), the poplar tent maker (*Ichthyura inclusa*), mourning cloak butterfly larvae (*Nymphalis antiopa*), the large aspen tortrix (*Choristoneura conflictana*), a leaf beetle (*Zeugophora scutellaris*), and the aspen blotch miner (*Phyllocnistis populiella*). The cottonwood twig borer (*Gypsonoma haimbachiana*) is particularly destructive, while several other borers may also do damage. Infestations of poplar gall midge (*Prodiptosis morrisoni*) and many species aphids are also responsible for damage (Dickmann and Stuart 1983, Demeritt 1990). In China, in Ningxia Autonomous Region, it was reported that 24 million trees were destroyed due to attack by the longicorn, *Anoplophora glabripennis* (Chinese National Report, IPC 1996). Several mechanisms are thought to operate to give hybrids more or less resistance relative to their parental species, but as a rule hybrid populations are centres of insect abundance (Whitham *et al.* 1996).

Other insect pests include:

– Satin moth	<i>Stilpnoia salicis</i>
– Viceroy butterfly larvae	<i>Gluphisia septentrionis</i>
– Gypsy moth	<i>Basilarchia archippus</i>
– Mourningcloak butterfly	<i>Lymantria dispar</i> [European and Asian varieties]
– Pandemis leafroller	<i>Nymphalis antiopa</i>
– Large aspen tortrix	<i>Pandemis pyrusana</i>
– Forest tent caterpillar	<i>Choristoneura conflictana</i>
– Poplar/willow borer	<i>Malacosoma disstria</i>
– Clear-winger poplar borer	<i>Chryptorhychus lathi</i>
– Poplar borer	<i>Panthrene robiniae</i>
– Bronze poplar borer	<i>Saperda calcerata</i>
– Scented willow sawfly	<i>Agrilus grandulatus lirogus</i>
– Phratora leaf Beetle	<i>Nematus salicis odoratus</i>
– Flea beetle	<i>Phratora californica</i>
– Williamette bailey	<i>Altica sp.</i>
– western willow lace bug	<i>Corythucha salicata</i>
– Cottonwood twig borer	
– Aphids	<i>Gypsonoma haimbachiana</i>

Further information about insect pest species of poplar can be found in Peterson, *et al.* 1996, Hiratsuka, 1987, Furniss and Carolin, 1977, Hepting 1971 and USDA Forest Service 1979. A useful Internet website is "<http://www.cas.psu.edu/docs/CASDEPT/PLANText/poplar.html>".

The fungi associated with *Populus* species are tremendously diverse. More than 250 species are known to be associated with the decay of *P. tremuloides* alone (Lindsey and Gilbertson 1978). Only fungi associated with sections *Populus*, *Aigeiros*, and *Tacamahaca* have been studied to any extent, and “virtually nothing is known” of those associated with other sections (Newcombe 1996).

The five most damaging or potentially damaging diseases of *Aigeiros* and *Tacamahaca* are:

- Melampsora leaf rust (*Melampsora* spp.) which, while causing only moderate levels of mortality in plantations, can cause volume growth reduction of up to 65% (Widin and Schipper 1981). The situation is now becoming serious in Europe as variability in *Melampsora larici-populina* has been reported and interspecific hybrid trees selected for complete resistance to this fungus are now affected by new races of this pathogen (Pinon 1992a,b, Pinon, 1995, Pinon and Frey 1997).
- Marssonina anthracnose or leaf spot (*Marssonina* spp.), affecting *Aigeiros* species and some of their intersectional hybrids in particular, and causing an estimated 16% loss from production plantations in Italy (Thielges 1985).
- Bacterial canker (*Xanthomonas populi* Ridé), causing serious damage to non-native *Aigeiros* and *Tacamahaca* species planted in Europe (Thielges 1985).
- Dothiciza canker (*Discosporium populeum*), of minor importance in North America, but causing wide-spread, heavy losses to *P. × canadensis* clones in Europe (Waterman 1957, Thielges 1985).
- Septoria leaf spot and canker (*Septoria musiva* Peck), which is generally limited to a leaf spot in native stands but is particularly damaging to hybrids, and has prevented the general use of most *P. × canadensis* clones in Canada, the United States and Argentina (Thielges 1985).

Members of the *Populus* section are most likely to be affected by:

- Hypoxylon stem canker [*Hypoxylon mammatum* (Whal.) Miller] is broadly distributed on host species in section *Populus*, but only becomes a disease problem in certain areas (Manion and Griffin 1986, Newcombe 1996). It sometimes causes cankers on *P. trichocarpa* in Europe (Terrasson *et al.* 1988) and on various hybrid clones in North America (Ostry and McNabb 1986).
- White-rotting fungus (*Phellinus tremulae* (Bond.) Bond. & Borisov.) causes serious decay in aspens (Thomas *et al.* 1960), although the mechanism of resistance by members of *Aigeiros* and *Tacamahaca* to decay is unknown (Newcombe 1996).

Various poplar species and hybrids display well-developed adaptations to environmental stress, but in particular to drought, flooding, salinity, cold, and atmospheric pollutants such as ozone (Blake *et al.* 1996, Neuman *et al.* 1996). It is also noted that *P. canadensis* shows stability against strong winds.

Many mammals feed on the bark, leaves and roots of *Populus*, notably snowshoe hares (*Lepus americanus*), beaver (*Castor canadensis*), porcupine (*Erethizon dorsatum*), pocket gophers (*Thomomys bottae*), and opossum (*Trichosurus vulpecula*) (Edwards 1978, Bryant 1981, Cantor and Whitham 1989, Basey *et al.* 1990). Ungulates such as deer, moose and elk (*Cervus elaphus*) not only browse on shoots and new sprouts, but also damage bark by chewing and rubbing with their antlers (Romme *et al.* 1995). Cattle and sheep also browse on regeneration and cause root damage to existing trees when allowed to range through stands (Cooper and Van Haverbeke 1990, Perala 1990). While little is known about the response of mammals to patterns of hybridisation in *Populus*, there is tremendous variation in feeding preference

among hybrids and individual clones. It is suspected that this is due to variation in concentrations of phenolic glycosides which are known to be defensive toward mammals (Whitham *et al.* 1996).

Mice and voles can cause severe damage in young plantations (DeBell 1990). Many species of birds thrive in poplar forests, and a few can cause damage through their feeding. The ruffed grouse and the sharp-tailed grouse feed on aspen buds, and the ruffed grouse also feeds on the leaves during the summer months. Red-breasted and yellow-bellied sapsuckers may scar trees with drill holes as they forage for bark insects (Fowells 1965).

The riparian cottonwoods are one of the most productive and sensitive components of riparian ecosystems in western North America. The number of vertebrate species associated with these communities is four times higher than the numbers associated with spruce-fir, lodgepole pine, or Douglas-fir communities, yet human activities result in the loss of over 100 000 ha of riparian habitat each year (Finch and Ruggiero 1993). While riparian ecosystems occur on less than 1% of the western North American landscape, they provide habitat for more bird species than all other vegetation types combined (Knopf *et al.* 1988). Zones of hybridisation are thought to be centres of biodiversity (Whitham *et al.* 1996), representing a refugium for insect species (Whitham 1989), and thus a superior habitat for insectivorous birds (Martinsen and Whitham 1994, Dickson and Whitham 1996).

## 8. Summary

The wide distribution of *Populus* throughout the Northern Hemisphere represents an important and valuable component of many forest ecosystems and great potential for domestication. Evolution of the genus has been characterised by divergence into various sections, which offer even more opportunities for novel genetic combinations through hybridisation. Gene flow within the range of the individual species is usually very high, with populations distinguished only by their adaptive response to environmental selection pressures.

While initial establishment is by seeds, which may travel long distances to invade newly disturbed areas, the maintenance of populations often relies on poplar's ability to reproduce vegetatively. Plantation culture of poplars has exploited this trait, and most breeding programs are characterised by deployment of selected clones. Clonal variation is high for yield traits, as well as disease resistance and wood quality.

In North America, *P. tremuloides* is the most widely distributed tree species and an important component of many forest types. The riparian cottonwoods, from sections *Aigeiros* and *Tacamahaca*, play an important role in the maintenance of complex riparian communities, in addition to their importance in plantation culture. Although North American poplar communities are still largely intact, small populations on the edge of species' ranges are in need of conservation. Of even greater concern is the erosion of genetic resources, particularly for *P. nigra*, that has resulted from human activity in Europe.

Poplar is ideally suited as a model organism for understanding growth processes in forest trees. It has and will undoubtedly continue to be a target for domestication and forest management in many parts of the world.

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## APPENDIX

### Genetic Transformation of Poplars

Poplar is the first forest tree species to have been transformed (Fillatti *et al.* 1987). Since that time, a number of different transformation procedures have been optimised on poplar species in many laboratories. These allow for the easy recovery of transgenic poplar plants (Jouanin *et al.* 1993). *Agrobacterium*-mediated transformation appears to be the most widely used vector for DNA transfer in poplar (Leplé *et al.* 1992). The first reports on poplar transformation deal primarily with the introduction of marker genes: the GUS gene or genes conferring selectable traits such as resistance to antibiotics or to herbicides (Brasiliero *et al.* 1992). Since 1990, an increasing number of studies have focused on the expression of genes potentially able to modify agronomic traits, for example: tolerance to insect attack (Robinson *et al.* 1994, Leplé *et al.* 1995, 1998), control of flowering (Weigel and Nilsson, 1995), modification of wood quality through altered lignin content and composition (Baucher *et al.* 1996, Van Doorselaere *et al.* 1995), improvement to oxidative stress tolerance (Strohm *et al.* 1995). Until recently, most of the results have been obtained using juvenile material grown under controlled conditions, however, an increasing number of field trials are now being set up with transgenic poplars in order to validate results obtained in the greenhouse. Moreover, these field evaluations will answer a number of questions concerning the spatial and temporal stability of transgene expression in mature trees that are subject to a natural changing environment (Pilate *et al.* 1997).

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## SECTION 5

### SITKA SPRUCE (*PICEA SITCHENSIS* (BONG.) CARR.)

#### 1. General Information

This consensus document addresses the biology of Sitka spruce (*Picea sitchensis* (Bong.) Carr.). Sitka spruce is an ecologically important species of the north temperate coastal rain forest of western North America. It is a valuable species for both pulp and lumber. Its wood offers unique qualities for manufacture of the highest quality sounding boards and tops for many musical instruments. As well, an outstanding strength-to-weight ratio made Sitka spruce strategically important during both World Wars for construction of aircraft (Brazier, 1987). While its natural range is not extensive and the species' economic importance ranks far below that of other western conifers, it is a keystone species in some of the most productive ecosystems of North America, particularly in the Queen Charlotte Islands of British Columbia (Peterson *et al.*, 1997). Outside its natural range, Sitka spruce has played an important role in plantation forestry, particularly in Northern Europe (Hermann, 1987). In Great Britain, the species now accounts for almost 70% of the annual conifer planting stock (Malcolm, 1997) and plantations cover over 20% of the forest/woodland area (Cannell and Milne, 1995). Sitka spruce is also a primary plantation species in Brittany, where productivity of stands is similar to that in Britain (Vaudelet, 1982; Serrière-Chadoeuf, 1986; Guyon, 1995).

The general biology of Sitka spruce is described in the context of the species' role in natural forests and its domestication in planted stands. Taxonomic and evolutionary relationships with other *Picea* species are described. Reproductive biology is described with a focus on aspects of mating system, gene flow, seed production and natural stand establishment. The current knowledge of genetic variation within the species is reviewed, highlighting the importance of variation patterns and the potential for improvement by means of recurrent selection breeding strategies. Biological diversity and ecological interactions with higher and lower flora and fauna are discussed. Domestication and operational breeding activities are reviewed. While Sitka spruce reforestation is currently based on seed propagation, vegetative propagation of rooted cuttings is well advanced, and somatic-embryogenesis techniques are available making it a logical target for implementation of transgenic biotechnologies and the use of cloning in both breeding and deployment strategies.

This document was prepared by the lead country, Canada. It is intended for use by regulatory authorities and others who have a responsibility for conducting assessments of transgenic plants proposed for commercialisation, and by those who are actively involved with genetic improvement and intensive management of this species.

#### 2. Taxonomy and Natural Distribution

##### A. Taxonomy and nomenclature

Sitka spruce (*épinette Sitka* in French Canada, *épicéa Sitka* in France, *Sitkafichte* in Germany) is one of about 40 species of the genus *Picea* A. Dietr. (family Pinaceae) distributed throughout the cooler parts of the North Temperate Zone and higher elevations in the south. It is also one of 7 species native to North America and 5 native to Canada (Farrar, 1995). There is lack of agreement among taxonomists regarding

the subdivision of the genus *Picea* (Schmidt-Vogt, 1977). Most early taxonomists suggested dividing the genus into three sections: Eupicea (or Morinda), Casicta, and Omorika. Mikkola (1969) recommended recognition of only two sections: Abies and Omorika. After extensive crossability studies, Fowler (1983, 1987) has suggested that the section Omorika be further divided into two subsections: Omorikoides and Glaucoides, with Sitka spruce assigned to the latter, together with White Spruce and Engelmann spruce.

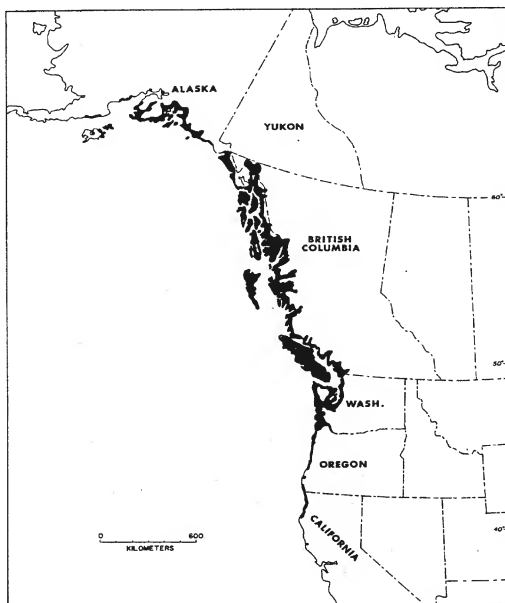
Originally introduced to Great Britain by David Douglas in the early 1800s as *Pinus menziesii*, the species was described soon after by the French botanist Bongard as *Pinus sitchensis*, referring to the origin of his specimens, Sitka Island, Alaska (now known as Baranoff Island) (Peterson *et al.*, 1997). Carrière later recognised the species as a member of genus *Picea*, and the species is now well recognised as *Picea sitchensis* (Bong.) Carr. Common names are numerous, including airplane spruce, coast spruce, Menzies spruce, silver spruce, tideland spruce, western spruce, and yellow spruce. A famous rare mutant form found on the Queen Charlottes is known as golden spruce (Peterson *et al.*, 1997).

Introgressive hybridisation between Sitka and White Spruce (*Picea glauca* (Moench) Voss) occurs in sympatric areas in north-western British Columbia and Alaska, with the hybrid known as *Picea* × *lutzii* Little (Little, 1953; Daubenmire, 1968; Roche, 1969; Hanover and Wilkinson, 1970; Copes and Beckwith, 1977; Yeh and Arnott, 1986; Woods, 1988). Introgressive hybridisation between white and Englemann spruce (*Picea englemannii* Parry ex Engelm.) is common where the two are sympatric in western Canada, Montana and Wyoming, and the hybrids have given rise to the variety *Picea glauca* var. *albertiana* (S. Brown) Sarg., commonly known as "interior spruce" (Roche, 1969; Roche *et al.*, 1969; Daubenmire, 1974). Sitka spruce hybridises with Englemann spruce through controlled crosses (Johnson, 1939; Roche, 1969; Jeffers, 1971; Fowler and Roche, 1977; Kiss, 1989), and there is evidence suggesting that hybrids among Sitka, white and interior spruce also occur naturally (Woods, 1988; Sutton *et al.*, 1991a, b, 1994; Coates *et al.*, 1994; Grossnickle *et al.*, 1996a, b). Several horticultural varieties, most of them dwarf phenotypes, have been recognised (Krüssmann, 1985; Griffiths, 1994).

## B Natural distribution

The natural range of Sitka spruce spans a narrow strip on the north Pacific coast of North America, extending for 2 900 km from 61°N latitude in south-central Alaska to 39°N in northern California. Throughout this tremendous north-south range, Sitka spruce is a coastal species, occupying islands of the Alexander Archipelago in Alaska and the Queen Charlotte Islands in British Columbia, and, with the exception of river valleys, they rarely reach more than a few kilometers from the coast along a narrow mainland strip. The southern limit of the species is an isolated population in Mendocino County, California (Harris, 1978). The natural range of Sitka spruce is illustrated in the map given in Figure 3.5.

Figure 3.5 The natural range of Sitka spruce



Source : Harris, 1978

### C. Evolution and migrational history

Conifers probably originated around the periphery of the north Pacific basin (Li, 1953). Fossil records indicate that divergence of modern genera in Pinaceae occurred some 135 million years ago during the late Jurassic or early Cretaceous period (Florin, 1963). Based on comparative immunological studies, Prager *et al.* (1976) have suggested that *Picea* was among the first genera to emerge. Although not supported by fossil evidence, Wright (1955) suggested eastern Asia as the likely origin of *Picea*, based on the abundance of species and particularly the presence of *Picea koyamai* Shirasawa, which he felt was a primitive species. *Picea* is then thought to have migrated to North American in one or more waves of migration via a land bridge between Siberia and Alaska (Wright, 1955).

Phylogenetic relationships within coniferous genera are commonly interpreted from species crossability studies, where it is assumed that the more related are two species, the more easily they can be crossed (Wright, 1955; Critchfield, 1975). The close phylogenetic relationship between the north-western American “white” spruces (Sitka, white, and Engelmann spruce) and the eastern Asiatic *Picea jezoensis* (Sieb et Zucc.) Carr. (Wright, 1955; Roche and Fowler, 1975) supports this theory, at least for the members of the subsection *Glaucoides* in section *Omorika*, and suggests that speciation occurred after their arrival in North America.

The fossil record of *Picea* during the Pleistocene era in North America is incomplete, but it is believed that many conifer populations were fragmented and isolated in various refugia during the glacial period (Critchfield, 1984). Sitka spruce probably occupied roughly its present-day range before glaciation, surviving at higher elevations on hills and mountains, and reoccupying lower areas of the long coastal strip in British Columbia and Alaska soon after (Daubenmire, 1968; Page and Hollands, 1987).

### 3. Reproductive Biology

#### A. Reproductive development

Sitka spruce is monoecious. Development of the reproductive structures follows a 2-year cycle typical of most conifers in the northern hemisphere, other than *Pinus* species and members of the Cupressaceae family (Owens and Blake, 1985). Bud scales are initiated at the terminal apex and at newly initiated axillary apices within the enlarging vegetative buds, from about mid-April (Owens and Molder, 1976a; Cannell and Bowler, 1978). Apices differentiate as vegetative, pollen cone, or seed cone buds around mid-July, at the cessation of shoot elongation. Pollen cones typically develop from small axillary apices on vigorous distal shoots or terminal apices on less vigorous, proximal shoots. Seed cones usually develop on distal axillary positions on vigorous shoots or from smaller terminal apices on less vigorous shoots (Moir and Fox, 1975a; Owens and Molder, 1976b).

Pollen cone bud development is complete, although meiosis has not occurred before they become dormant at the end of October. Seed cones also do not undergo meiosis prior to becoming dormant in late November (Owens and Molder, 1976b). By the time buds become dormant, all microsporophylls, microsporangia, bracts and functional ovuliferous scales, and leaves have been initiated. The overwintering seed cone, pollen cone, and vegetative buds are small and similar in shape: broadly conical, greenish-brown and covered in a bloom of light grey resin (Moir and Fox, 1975a; Eis and Craigdallie, 1981).

Reproductive and vegetative buds break dormancy at about the same time, in response to photoperiod, while subsequent development is regulated by temperature. Meiosis and subsequent development of pollen occur immediately, followed by maturation of the megagametophyte (Moir and Fox, 1975b; Owens and Molder, 1980). Flushing of reproductive buds precedes that of vegetative buds, and pollen is released over a one-week period, in late-April on Vancouver Island (Owens and Molder, 1980) and by mid-May in Scotland (Moir and Fox, 1975a). The pollen enters receptive seed cones and adheres to the sticky micropylar arms. A week later, a “pollination drop” draws the pollen into the micropyle (Owens and Blake, 1984). Fertilisation occurs 4 to 5 weeks later, and embryo development is completed in mid-August. Without fertilisation, no embryo is formed and the megagametophyte tissue degenerates, leaving a normal-sized, but empty seed (Owens and Molder, 1980).

#### B. Mating system and gene flow

Sitka spruce is a wind-pollinated, monoecious species, and outcrossing is by far the most prevalent mating system (Cottrell and White, 1995). Self pollination occurs to some degree, as the period of pollen release and female receptivity coincide for an individual tree (Owens and Molder, 1980; El-Kassaby and

Reynolds 1990). Female strobili are usually found at the ends of primary branches in the mid- to upper-crown, while males are more prevalent at the ends of secondary branches lower in the crown (Tompsett, 1978; Philipson, 1997), although the effectiveness of this zonation against selfing is questionable (Nienstaedt and Teich, 1972). In the open-grown conditions in a seed orchard, the outcrossing rate was greatly reduced for seeds produced in the lower crown (Chaisurisri *et al.*, 1994). The two-step pollination mechanism, whereby pollen is collected in the sticky micropylar arms over the receptive period, and only then drawn *en masse* by the pollination drop, ensures that pollen from many sources has a chance to fertilise any given ovule (Owens and Blake, 1984; Runions *et al.*, 1995).

Gene flow in *Picea* is mediated by small pollen grains, 70–85  $\mu\text{m}$  at their widest point (Eisenhut, 1961), whose bladdery wings make them well-adapted for aerial transport (Di-Giovanni and Kevan, 1991). Various studies of pollen dispersal in conifers indicate that over 90% of the pollen comes to rest less than 100 m from the source (Wright, 1976). Nevertheless, conifer pollen may remain viable for several days and a substantial quantity may travel great distances (Lindgren *et al.*, 1995; Lindgren and Lindgren, 1996). Gregory (1973) cites reports that pollen of *Pinus* and *Picea* may travel as far as 600 to 1 000 km, and several authors have concluded that isolation distances of less than 1 km often have little impact on contamination rates in conifer seed orchards (see review by Di-Giovanni and Kevan, 1991). While pollen dynamics are not well known in Sitka spruce, a recent study of pollen dispersal dynamics in a black spruce seed orchard indicated that "large amounts" of pollen rose to a height of 300 m above ground level (Di-Giovanni *et al.* 1996). At a steady wind speed of  $5 \text{ m}\cdot\text{s}^{-1}$ , the authors calculated that spruce pollen reaching this altitude would drift about 47 km.

### C. Seed production

Sitka spruce begins to produce seed at 20 to 25 years of age, with heavy crops occurring at intervals of 3 to 5 years (Malcolm, 1987; Philipson, 1987b). Crop intervals are somewhat longer, 5 to 8 years, in the northern part of the range (Harris, 1969). It is a prolific seed producer and, in a good seed year, an old-growth stand may produce as much as 14.5 kg of seed per hectare (Peterson *et al.*, 1997). The seeds themselves are small, and average cleaned seed weight is about 2.2 g/1000 seeds (Safford, 1974).

Initiation and duration of seed dispersal are weather and site dependent. The mature cones open as they lose moisture and the scales flex in dry weather, re-closing during wet periods. Seed dispersal begins in the fall, with over 70% of the seeds dispersed within the first 6 weeks, 90% by February, and the remainder released over the next growing season (Ruth, 1958; Harris, 1969). The seeds are winged and wind-dispersed. The actual distance reached from the source depends on several factors, including height and position of the seed source, local topography and wind conditions (Harris, 1967, 1978). While 80% of the seed usually falls within 30 metres of the parent tree, some may travel up to several hundred metres (Mair, 1973).

### D. Natural regeneration

Sitka spruce seeds exhibit weak dormancy, and both the rate and total amount of germination can be increased by exposure to low temperatures under moist conditions, *i.e.*, cold stratification (Pintaric, 1972; Gordon *et al.*, 1976; Gosling, 1988; Chaisurisri *et al.*, 1992; Jinks and Jones, 1996). While not always employed in nursery practice, moist-chilling of Sitka spruce seed is often beneficial, particularly when moisture content and temperature are carefully controlled (Gosling and Rigg, 1990; Jones *et al.*, 1993; Jones and Gosling, 1994), and stratified seed will tolerate redrying (Jones and Gosling, 1990; Jinks and Jones, 1996; Poulsen, 1996). In the wild, Sitka spruce seeds normally germinate the following spring, as soon as soil surface temperatures are warm enough and provided there is adequate moisture.

Unlike its highly shade tolerant associates western hemlock and western red cedar that can germinate and survive on organic seedbeds, Sitka spruce is more restricted in its seedbed and light requirements and tends to be more disturbance dependent (Taylor, A.H., 1990; Peterson *et al.*, 1997). Sitka spruce regenerates naturally on landslides, newly exposed alluvial sites, and openings created by windthrow. Regeneration cutting systems in Sitka spruce must generate sufficient ground exposure and disturbance, by clear cutting, shelterwood or seed-tree methods (Harris and Johnson, 1983; Weetman and Vyse, 1990). Regarding thinning operations in mixed western hemlock, Sitka spruce stands will normally favour regeneration of the hemlock over the spruce, particularly in older stands where thinning intensity is light (Deal and Farr, 1994). Dense natural regeneration has been more commonly observed after harvesting of first-generation planted stands in Britain, with up to several hundreds of thousands per hectare and requiring spacing (Nelson, 1991; Adam and Berg, 1996).

#### E. Vegetative reproduction in nature

Vegetative reproduction of Sitka spruce is rare under natural conditions or in plantations, although layering can occur on moist sites (Cooper, 1931; Roche and Fowler, 1975). Rooting is most likely to occur when lower branches of open-grown trees come in contact with the ground and become covered by soil or organic materials on the edges of bogs or near the timber line (Harris, 1978).

#### 4. Crosses

Potential crosses with Sitka spruce are summarised in Table 3.7. Natural hybrids between Sitka and White Spruce were first collected in North America by H.J. Lutz on the Kenai Peninsula, Alaska, and given the name *Picea × lutzii* by E.L. Little (1953). Before this, white-Sitka spruce hybrids had been observed in Denmark as a result of natural crossing between adjacent plantations (Thaarup, 1945; Bornebusch, 1946). Populations resulting from introgressive hybridisation have since been documented in the Skeena, Nass, and Bulkley river valleys in British Columbia where the two species are sympatric (Daubenmire, 1968; Roche, 1969). The hybrid has frequently been made artificially with parents from outside the sympatric area (Fowler, 1987), often in the hope of imparting the resistance of White Spruce to the white pine weevil. The degree of cold hardiness of the hybrid is related to the proportion of White Spruce germplasm (Ying and Morgenstern, 1982), and growth performance of the hybrid depends greatly on the origin of the parents (Sheppard and Cannell, 1985). Though *Picea breweriana* and *Picea sitchensis* can cross successfully, *P. breweriana* has a very small range that is rarely, if ever, sympatric with *P. sitchensis* given that the populations are separated by elevation. The category of “easily crossed, probably occurring naturally” indicates species that readily cross with *P. sitchensis* if grown together in artificial plantations, but are not naturally sympatric.

Table 3.7 Species cross compatibility with Sitka spruce

Species	Origin	References
<i>Commonly occurring in sympatric range</i>		
<i>P. glauca</i> (Moench) Voss. = <i>Picea × lutzii</i> Little	Canada, Northeast USA	Daubenmire, 1968; Roche, 1969; Fowler, 1987; Woods, 1988
<i>Easily crossed, probably occurring naturally</i>		
<i>P. englemannii</i> Parry ex Engelm.	Canada, Western USA	Johnson, L.P.V. 1939; Roche, 1969; Jeffers, 1971; Fowler and Roche, 1977; Kiss, 1989
<i>P. breweriana</i> Wats.	Northwest USA	Langner 1952
<i>P. mariana</i> (Mill.) B.S.P.	Canada, Northern USA	Fowler, 1983
<i>Successful crosses</i>		
<i>P. jezoensis</i> (Sieb. & Zucc.) Carr.	Japan	Wright, 1955; Roulund, 1969
<i>P. omorika</i> (Pancic) Purkyne	Western Serbia, Eastern Bosnia	Johnson, 1939; Langner, 1959; Roulund, 1971; Geburek and Krusche, 1985
<i>Possible crossability</i>		
<i>P. abies</i> (L.) Karst (= <i>P. excelsa</i> (Lam.) Link)	Northern, Central, Eastern Europe	Langner, 1952
<i>P. likiangensis</i> (Franch.) Pritz.	China	Roche and Fowler, 1975
<i>P. pungens</i> Engelm.	Western USA	Roche and Fowler, 1975
<i>P. wilsonii</i> Mast.	China	Roulund, 1969

## 5. Genetics

### A. Cytology

Sitka spruce vegetative cells normally have  $2n = 24$  chromosomes (Burley, 1965b; Fox, 1987), although some trees exhibit a small 13th pair (Moir and Fox, 1972; Kean *et al.*, 1982). These supernumerary or B-chromosomes seem to be restricted to provenances in the southern half of the species range (Moir and Fox, 1977), but have not been associated with any detectable effect on growth (Moir and Fox, 1976).

### B. Genetic variation

#### *Population-level variability*

Before 1970, information on population variation of Sitka spruce was only available from small studies with limited sampling. Even in these first limited trials, there was strong evidence of clinal variation for many traits, associated with latitude, elevation, and distance from the coast (Burley, 1965a; Roche and Fowler, 1975). In 1969/70, an extensive sampling of seed sources from across the entire range was organised by the International Union of Forest Research Organizations (IUFRO). Ten of these sources were widely planted in field tests in North America and many European countries (O'Driscoll, 1978).

Clinal variation patterns are expressed for phenological traits such as cessation of growth (Lines and Mitchell, 1966; Pollard *et al.*, 1975; Kraus and Lines, 1976), and is greater among provenances than within

(Falkenhagen, 1977; Deleporte, 1984). Southern coastal provenances produce up to 100% more height growth than northern inland sources (Cannell, 1974; Cannell and Willett, 1975; Cannell and Willet, 1976). While southern sources grow faster, they are more susceptible to frost damage, particularly in the nursery (Magnesen, 1986; Lines, 1987b; McKay, 1994). Provenance trials in the former Federal Republic of Germany showed a north-south trend in growth, with latitude accounting for over 80% of the among-provenance variation (Kleinschmit, 1984). Results of a 19 year provenance trial in Ireland demonstrated that the most productive provenances of Sitka Spruce for the mild, coastal conditions in Ireland originated from southern Washington and northern Oregon (Thompson and Pfeifer, 1995).

Ecotypic variation related to bioclimatic and physiographic factors has been demonstrated among provenances for seed and cone traits (Falkenhagen, 1978; Falkenhagen and Nash, 1978). Even for growth traits that normally exhibit clinal variation patterns, substantial variation may be present at the microgeographic level, attributable to such local site factors as slope and aspect (Campbell *et al.*, 1989). Variation in biochemical composition appears to be clinal for sources from Alaska to north Washington, while more southerly sources show no geographic trends, perhaps reflecting the post-glacial recolonisation of northern parts of the range (Forrest, 1975b, 1980; Wellendorf and Kaufmann, 1977).

Population differences have also been demonstrated for susceptibility to insect attack. Provenances from Kitwanga (inland Skeena River) and Big Qualicum (SE Vancouver Island) suffer less damage from the white pine weevil (Alfaro and Ying, 1990; Tomlin and Borden, 1994; Ying and Ebata, 1994). Density of green spruce aphids attacking a provenance test in northern Ireland was related to latitude of seed origin, with southern provenances especially susceptible (Day, 1984). Lignified stone cell masses in spruce bark are considered an important physical defence against insects and fungi, and there is a clinal increase in bark lignin with increasing latitude of provenance origin (Wainhouse and Ashburner, 1996).

In contrast to many other characters, geographic variation at polymorphic allozyme loci appears to be weak. In a study of the 10 IUFRO provenances, only 8% of the diversity at polymorphic loci was due to differences among populations, whereas 92% resided within populations (Yeh and El-Kassaby, 1980).

### *Individual-level variability*

While variation among provenances is important in determining the risks and benefits of transferring seed sources, genetic improvement from mass selection relies primarily on variation within-populations as the source of genetic gains. For productivity traits, on average, 40% of the genetic variation for Sitka spruce is at the provenance level, while 60% is within provenance (Fletcher, 1992).

Estimates of narrow-sense heritabilities for height growth during the first six years was around 25% for open-pollinated progeny of randomly selected trees in a single population (Samuel and Johnstone, 1979), while another study estimated heritability at over 40% for height after eight years (Biot and Christophe, 1983). Other quantitative estimates of additive genetic variation in growth traits have also varied widely (Falkenhagen, 1977; Yeh and Rasmussen, 1985; Samuel, 1991), but narrow-sense heritability is normally more than sufficient for individual tree selection and breeding to be effective, particularly when provenance and family performance are combined in a multiple-trait selection index (Christophe and Biot, 1983). Height superiority of individual trees is not necessarily associated with production of "free growth", although progeny of plus-trees have been noted to improve their height rankings over the first six years on better sites by production of free growth (Cannell and Johnstone, 1978).

Perhaps the most precise estimates of genetic variance for Sitka spruce have been reported for a 7-tree diallel cross, planted at two test sites in Scotland and Wales (Samuel *et al.*, 1972; Samuel, 1991). In this experiment, genetic variation for height growth, although under some additive genetic control, was predominantly controlled by non-additive effects, whereas diameter was only controlled by additive

effects. Practically all the variation in monoterpene composition for these crosses was attributable to additive genetic effects, and reflected the differences in parental means in additive combination with little significant deviation due to specific combining ability or reciprocal effects (Forrest and Samuel, 1988).

The ease of vegetative propagation of Sitka spruce offers opportunities to capture additional gains earlier in the breeding cycle. Clonal selection has been demonstrated to be highly effective in Sitka spruce, for characters such as planting stock phenology, frost resistance (Nielsen and Roulund, 1996), root-growth potential (Deans *et al.*, 1992), early height and diameter growth, and branching habit (Cahalan, 1981). Clonal testing has also demonstrated that it is possible to select clones that combine good growth with high wood density (Costa e Silva *et al.*, 1994).

A provenance test in British Columbia demonstrated significant difference in susceptibility to white pine weevil, both among provenances and among families within provenances (Alfaro and Ying, 1990; Ying, 1991). Unfortunately, a study of variation within a resistant provenance found that taller families were more likely to be attacked (Alfaro *et al.*, 1993).

Variation in biochemical composition for trees within provenances is high (Forrest, 1975a, b; Wellendorf and Kaufmann, 1977), a trend that is also found for variation at polymorphic enzyme loci (Yeh and El-Kassaby, 1980). While southern provenances are generally less resistant to frost, there is sufficient variation within populations to select frost resistant, fast-growing individuals (Nicoll *et al.*, 1996).

Genotype-environment interactions are of concern to tree breeders who generally seek broad adaptability within bred material. A seedling test of provenances grown under controlled environments found that provenances near the centre of the range were more broadly adapted (Mergen *et al.*, 1974). A test of families originating from the latitudinal range of Sitka spruce and planted at eight locations in Britain found highly significant family-site interaction for six-year height, but found that above-average families could be selected that were broadly adapted (Johnstone and Samuel, 1978). A progeny test established at 3 locations in Denmark demonstrated genetic control of growth, stem form, wood density (pilotyn penetration), frost resistance and resistance to aphids, in addition to substantial genotype-environment interaction for growth characters (Jensen *et al.*, 1996). Another clonal test in Denmark found that 15% of the clones contributed over 50% of the GE interaction variance (Nielsen and Roulund, 1996).

### **C. Inbreeding depression and genetic load**

Self pollination in Sitka spruce has severe effects on seed set, early growth, and survival (Samuel *et al.*, 1972). Among those that survive, strong inbreeding depression continues with selfed individuals only 68% as tall as outcrossed trees at 15 years of age (Samuel, 1991). Inbreeding depression is also exhibited by progeny originating from seed collections in small stands (Phillips, 1984).

### **D. Breeding programs**

Breeding strategies for Sitka spruce generally utilise a system of progeny testing and recurrent selection for generation advancement, combined with clonal seed orchards for production of improved seed. Flowering of Sitka spruce grafts can be stimulated by means of various cultural treatments, particularly those involving gibberellin A<sub>4</sub>/7, and this has facilitated the turnover of breeding cycles (Philipson, 1985a, b, 1987a, 1992; Philipson *et al.*, 1990; Ross, 1991; Owens *et al.*, 1992). While most seed orchards currently in production were established by grafting cuttings from plus-trees, and their placement in cultivated field environments, some programs have also experimented with the management of containerised Sitka spruce orchards, with the possibility of vegetative multiplication of small quantities of seed by rooted cuttings (John and Mason, 1987; Philipson and Fletcher, 1990).

Breeding programs have been established in all the areas where Sitka spruce is an important plantation species. In British Columbia, where planting of Sitka spruce is severely limited due to risk of damage by the white pine weevil, the breeding plan emphasises weevil resistance. Currently, more than 250 open-pollinated families and 300 clones are included in weevil-resistance screening trials (King, 1994; King *et al.*, 1998). The intensive breeding program in Britain began in the early 70's (Fletcher and Faulkner, 1972), and is by far the most ambitious. From an initial 2 800 plus-tree selections, the breeding population now consists of 200 tested parents, subdivided into sub-populations targeted for different geographic areas (Faulkner, 1987; Fletcher, 1992; Malcolm, 1997). Breeding programs are also carried out in Denmark (Roulund, 1990) and in northern France (Deleporte and Roman-Amat, 1986), where Sitka spruce is an important component of plantation forestry operations.

### E. Conservation of genetic resources

Domestication of a key species such as Sitka spruce can influence diversity of genetic resources: (1) indirectly, by the method of seed collection, extraction, and storage, and by nursery and plantation culture; and (2) directly, by intentional selection to increase the frequency of genes for desirable traits (Chaisurisri and El-Kassaby, 1994; Morgenstern, 1996). The inadvertent loss of genes by natural processes and human activity can have negative consequences on the adaptability of populations and the potential for future gains from breeding. The need for gene conservation for a species can be assessed by evaluating (1) its current status of protection, (2) its frequency of occurrence, (3) the extent of its botanical range, (4) ease of natural regeneration, and (5) its representation in genetic testing and breeding programs (Lester, 1996). Using this approach, Yanchuk and Lester (1996) ranked the need of Sitka spruce for gene conservation as higher than some of its associates (Douglas-fir, western red cedar and western hemlock), but lower than others (mountain hemlock, amabilis fir, yellow-cedar and western white pine).

In the case of Sitka spruce, *in situ* conservation of genetic resources is practised by protection of ecological reserves, special areas, and parks (Pollard, 1995), and integrated with domestication activities that control the movement of seed, active management of existing stands to maintain biological diversity, and protection of isolated, small populations (Yanchuk, 1995). *Ex situ* conservation, by cryopreservation of germplasm, by off-site maintenance of populations in arboreta and clone banks, and by multi-population breeding strategies (Eriksson *et al.*, 1993; Namkoong, 1995), has been practised to a much lesser extent, although many provenances and families are now represented in field tests and seed bank collections (Edwards and El-Kassaby, 1993). Such "active" forms of gene management must be accelerated in preparation for response to rapid environmental and climate changes (Ledig and Kitzmiller, 1992; Yanchuk and Lester, 1996).

## 6. Ecology and associated species

### A. Habitat

In its natural range, Sitka spruce is primarily a coastal species, but may extend well inland where optimum environmental conditions occur (Harris, 1990). It is primarily a low elevation species and is uncommon at elevations above 500 m. It is, however, much less restricted by edaphic factors than by climate and physiography (Roche and Haddock, 1987).

### *Climate*

The natural range of Sitka spruce is restricted to an area of maritime climate with abundant moisture throughout the year, relatively mild winters and cool summers. Annual precipitation is high throughout the range, but somewhat greater in the north where summer precipitation as light drizzle and fog are frequent. Summer temperatures in these coastal habitats lack the extremes of more continental areas and, while

moderated by the ocean current of the north Pacific, decrease northward. Although winters are mild, accumulated heat input varies with latitude and probably accounts for much of the variation in productivity, soil development, and associated species (Harris, 1978).

### *Soils and site type*

While Sitka spruce grows on soil derived from a variety of parent materials, their best development is on deep, moist, well-aerated soils. Growth is poor on swampy sites. Sitka spruce commonly occupies alluvial soils along streams, coarse-textured soils, or soils with a thick accumulation of organic matter. In Alaska, it is a pioneering species on immature soils exposed by glacial retreat or uplift from the sea. Soils in the natural range are acidic, with pH values varying from 4.0 to 5.7 (Harris, 1978).

Sitka spruce is relatively nutrient demanding, particularly at young ages prior to crown closure (Miller and Miller, 1987). It is most productive on nitrogen rich soils, but also requires relatively large amounts of calcium, magnesium and phosphorus (Krajina, 1969). Nitrogen and phosphorus have been found to be limiting on sites in both British Columbia and Britain, and applications of fertilisers may be necessary for successful plantation establishment (McIntosh, 1981, 1983; Miller and Miller, 1987; Taylor, C.M.A., 1990; Taylor and Tabbush, 1990; Prescott and Weetman, 1994).

### **B. Synecology and associated species**

Sitka spruce occurs most commonly in mixed stands, usually associated with western hemlock (*Tsuga heterophylla*) (Harris and Johnson, 1983). Red alder (*Alnus rubra*) and black cottonwood (*Populus trichocarpa*) are associated throughout the range. Other associates vary with latitude: Douglas-fir (*Pseudotsuga menziesii*), Port-Orford-cedar (*Chamaecyparis lawsoniana*), western white pine (*Pinus monticola*), redwood (*Sequoia sempervirens*), and bigleaf maple (*Acer macrophyllum*) are limited to the south; shore pine (*Pinus contorta* var. *contorta*) and western red cedar (*Thuja plicata*) extend into south-east Alaska; while yellow cedar (*Chamaecyparis nootkatensis*), mountain hemlock (*Tsuga mertensiana*), subalpine fir (*Abies lasiocarpa*), and Sitka alder (*Alnus sinuata*) are limited to northern sites and higher elevations in the south (Harris, 1978). In Alaska and the Skeena, Nass, and Bulkley river valleys of British Columbia, Sitka spruce is associated with White Spruce (*Picea glauca*), and hybrid populations are found (Daubenmire, 1968; Roche, 1969). Pure stands of Sitka spruce are common on tidewater areas that receive quantities of salt spray, and in early succession situations following disturbance. Sitka spruce is an aggressive pioneer and, by itself or together with intolerant associates such as alder or cottonwood, will invade landslides, dunes, uplifted beaches and glaciated terrain.

Due to the latitudinal spread of the Sitka spruce range and the variation in precipitation and exposure, the species is a component of several ecological associations, characterised by available moisture and nutrient regimes. On the mid- to upper-slopes of the Queen Charlotte Islands, the western hemlock – Sitka spruce forest is typically associated with Alaska blueberry (*Vaccinium alaskaense*), red huckleberry (*V. parifolium*), ovalleaf huckleberry (*V. ovalifolium*), and several mosses (*Rhytidiadelphus loreus*, *Hylocomium splendens*, and *Mnium glabrescens*). On exposed coastal locations, pure stands of salt-tolerant Sitka spruce are associated with reed grass (*Calamagrostis nutkaensis*), salal (*Gaultheria shallon*) and finger moss (*Stokesiella oregana*). Sheltered alluvial sites find Sitka spruce and its associates western hemlock and red alder on grass meadows with *Trisetum cernuum*, *Gymnocarpium dryopteris*, *Hylocomium splendens* and *Leucolepis menziesii* (Roche and Haddock, 1987; Hanley and Hoel, 1996; Hanley and Brady, 1997).

Inland valleys and eastern slopes of coastal mountains tend to be drier. It is on these areas that Sitka spruce is most likely to be sympatric with White Spruce and hybridisation can occur. Within this drier zone, valley bottoms and mountain slopes typically support mixed conifer forests with ground vegetation

dominated by Alaska blueberry, ovalleaf huckleberry, red huckleberry, and rustyleaf menziesii (*Menziesia ferruginea*). Devil's club (*Oplopanax horridus*) appears within this zone on fluvial sites of the Skeena and Nass Rivers, and skunk cabbage (*Lysichiton americanum*) and salmonberry (*Rubus spectabilis*) are found on the driest sites in flat areas (Roche and Haddock, 1987; Harris, 1990).

### C. Competition and stand structure

While Sitka spruce is rated as tolerant to shade, it is less tolerant than its usual associate, western hemlock (Daniel *et al.*, 1979; Minore, 1979; Kobe and Coates, 1997), so that the general successional tendency is toward a western hemlock climax type, although few climax stands proceed to pure hemlock. As Sitka spruce is physically large, long-lived, and able to invade small openings resulting from windthrow, it is commonly maintained as a stand component, even under climax conditions. In south-east Alaska, mixed stands of hemlock and spruce are regarded as the climax stand type, with Sitka spruce regenerating on mineral soil mixtures exposed by windthrow and other disturbance, and hemlock seeding in on organic substrates (Harris, 1990; Deal *et al.*, 1991; Peterson *et al.*, 1997).

Sitka spruce is one of few conifer species that produce epicormic shoots along the stem. These shoots may originate from either dormant or adventitious buds (Stone and Stone, 1943) in response to light intensity (Isaac, 1940; Herman, 1964). Increasing exposure of stems to sunlight by thinning of stands will stimulate epicormic branching and affect the future quality of the trees (Farr and Harris, 1971).

### D. Ecosystem dynamics

Many abiotic factors interact with Sitka spruce in natural and planted forests, and some may cause significant damage. Windthrow is probably the most serious damaging agent, particularly in plantations of Sitka spruce that are established in Great Britain where shallow rooting on unfavourable soils and exposure to strong winds results in risk of instability (Miller, 1986; Coutts and Philipson, 1987; Mason and Quine, 1995; Malcolm, 1997). Elsewhere in Europe, planted Sitka spruce has suffered significant wind damage, but has proven more wind-firm than other conifers such as *Picea abies*, *Abies* spp. and *Pinus sylvestris* in France (de Champs *et al.*, 1983; Touzet, 1983), Denmark (Neckelmann, 1981) and Norway (Lohmander and Helles, 1987). In North America, Sitka spruce is considered less wind-resistant than *Pseudotsuga menziesii* and *Thuja plicata*, but more-so than *Tsuga heterophylla* and *Abies amabilis* (Minore, 1979).

While Sitka spruce is among the least fire-resistant species in coastal forests, wild fires are not a major cause of damage within the native range (Minore, 1979; Agee, 1990). On the other hand, Sitka spruce regeneration benefits rather more from slash burning than several of its conifer associates (Hawkes *et al.*, 1990; Otchere-Boateng and Herring, 1990). Frost heaving can cause severe damage, particularly to container seedlings planted on finer-textured soils (Shaw *et al.*, 1987; Goulet, 1995). Autumn frost is a particular problem in Great Britain, where faster-growing southern provenances are particularly susceptible (Redfern and Cannell, 1982; Lines 1987b; Nicoll *et al.*, 1996).

In the following table, other species interactions with Sitka Spruce are shown.

Table 3.8 Species Interactions with Sitka Spruce

Insects	
Common name	Agent
White pine weevil [Johnson, 1965; McMullen, 1976; Alfaro, 1982; Martineau, 1984; de Groot, 1985; Wallace and Sullivan, 1985; Hulme, 1986, 1987; McMullen <i>et al.</i> , 1987; Alfaro, 1989a,b; Hulme and Harris, 1989; McLean, 1989; Alfaro and Omule, 1990; Alfaro and Ying, 1990; Warkentin <i>et al.</i> , 1992; Fraser and Heppner, 1993; Alfaro, 1994; Fraser and Szeto, 1994; Hulme, 1994; Sahota <i>et al.</i> , 1994; Spittlehouse <i>et al.</i> , 1994; Tomlin and Borden, 1994; Alfaro <i>et al.</i> , 1995; Hulme, 1995; Alfaro, 1996a,b; de Groot and Zylstra, 1996; Tomlin <i>et al.</i> , 1996; and, Tomlin and Borden, 1997a,b; ]	<i>Pissodes strobi</i> ; The most serious economic insect pest of Sitka spruce in North America. Larvae tunnel down the inner bark of the shoot, killing the leaders. Not yet a pest where Sitka spruce is planted in Europe.
Green spruce aphid [Powell and Parry, 1976; Carter, 1977; Dixon, 1977; Koot, 1983; Evans, 1987; Nichols, 1987; Carter, 1989; Sutherland <i>et al.</i> , 1989; Finck <i>et al.</i> , 1990; Seaby and Mowat, 1993; Straw, 1995; Schwenke, 1972]	<i>Elatobium abietinum</i> ; a sap sucking insect, can result in mortality at high infestations levels
Bark beetles (Scolytidae) Spruce beetle Great spruce bark beetle [Bejer-Petersen, 1976; Lemperiere and Bailey, 1986; Fielding <i>et al.</i> , 1991; Kirkeby-Thomsen, 1992; Rose <i>et al.</i> , 1994, and Reynolds and Holsten, 1996]	<i>Dendroctonus rufipennis</i> ; feeds and breeds in galleries between bark and wood. <i>D. micans</i> (ditto)
Fungi	
Disease	Agent
Annosus root rot [Pratt, 1979a, b; and Morrison <i>et al.</i> , 1986]	<i>Heterobasidioion annosum</i> ; causes butt-rot and can lower yield and quality
Armillaria root rot [Boullard and Gaudray, 1975; Redfern, 1978; and Morrison, 1981]	<i>Armillaria mellea</i> complex; may kill younger trees
Laminated root rot [Nelson and Sturrock, 1993; and Thies and Sturrock, 1995]	<i>Phellinus weirii</i> ; butt decay that may kill younger trees
Rhizinia root rot [Phillips and Young, 1976; Gregory and Redfern, 1987; and Callan, 1993]	<i>Rhizinia undulata</i> ; can affect young seedlings and pole sized trees

For other rust fungi, stem decay, nursery moulds and diseases of seed and cones see: [Gregory and Redfern, 1987; Sutherland <i>et al.</i> , 1987, 1989 and Sutherland and Hunt, 1990]	
<b>Animals</b>	
Common name	Species name
Sitka spruce stands provide cover for many species of fish (salmon and trout species), mammals and birds [Hartman and Brown, 1988; and Staines <i>et al.</i> , 1987]	
Black-tailed deer [Sullivan <i>et al.</i> , 1990] Red deer Sika deer Roe deer Fallow deer [Welch <i>et al.</i> , 1987, 1991, 1992; Hannan and Whelan, 1989 and de Jong <i>et al.</i> , 1995]	<i>Odocoileus hemionus columbianus</i> ; heavy numbers may cause browsing damage <i>Cervus elaphus</i> <i>C. nippon</i> <i>Capreolus capreolus</i> <i>Dama dama</i> All may cause damage by bark stripping and browsing
Porcupine [Sullivan <i>et al.</i> , 1986]	<i>Erethizon dorsatum</i> ; may feed cause slight damage
Red squirrel [Syme, 1985]	<i>Tamiasciurus hudsonicus</i> ; damages shoots in removing cones
Seed-eating birds	Many bird species commonly eat quantities of seed, as well as insects associated with Sitka spruce.

### E. Symbiotic Relationships - Mycorrhizae

Relatively little research has been done on mycorrhizas of Sitka spruce, although results from forest trials show that inoculation with selected mycorrhizal fungi can give significant early growth effects (Walker, 1987). For example, seedlings inoculated with E-strain fungi, the dominant mycorrhizal fungi of nurseries, were smaller than those inoculated with either *Thelephora terrestris* or *Laccaria laccata* (Thomas and Jackson, 1983). The dominant mycorrhizal fungus in the nursery, the 'E-strain', decreases in frequency with age after planting out. Some mycorrhizal types are found at all forest sites in Britain: of these types, *Thelephora terrestris* has been found on all age classes of Sitka spruce. Other mycorrhizal species recorded on Sitka spruce include *Amanita rubescens*, *Laccaria amethystea*, *Lactarius hepaticus*, *L. tabidus*, *L. turpis* and *Russula ochroleuca* (Thomas *et al.*, 1983). The successions of fruit bodies of mycorrhizal fungi under differently aged British plantations of Sitka spruce were determined to be *Laccaria/Paxillus-Inocybe-Cortinarius-Lactarius* (Dighton *et al.*, 1986).

Eighty-four potentially mycorrhizal macrofungi have been recorded with Sitka spruce in Scotland. They derive primarily from the native flora of birch and pine and many are fungi with a wide host range. Specific mycorrhizal associates do not occur. The saprotrophic macrofungi are species that are common in a range of vegetation types (Alexander and Watling, 1987). In nurseries in the Irish Republic, *Piceirhiza horti-inflata* was the most frequent mycorrhizal association during the first year of growth but appeared to be replaced by *Hebeloma* sp. and *Amphinema byssoides* in 2-year-old seedlings. There was a greater diversity of mycorrhizas on container-grown seedlings, which included *Thelephora terrestris*, *Hebeloma* sp. and *Piceirhiza guttata* (Grogan *et al.*, 1994). Sitka spruce trees in a plantation established in Normandy in 1956 were affected by *Armillaria mellea* root rot and it is suggested that the formation of mycorrhizae on *P. sitchensis* in France (where the tree is an exotic) is in some way incomplete and affords inadequate protection against *A. mellea* (Gaudray, 1973).

The ability of six ectomycorrhizal fungi (*Thelephora terrestris*, *Hebeloma crustuliniforme* strains Siv and 81a, *Paxillus involutus*, *Laccaria laccata* and *Lactarius rufus*) to form mycorrhizas on plantlets of Sitka spruce derived from somatic embryos was investigated by Sasa and Krogstrup (1991). Mycorrhizal synthesis was achieved only on the oldest plantlets during the third week after inoculation. The rate and development of mycorrhizal formation varied according to the fungal species, with infection by *T. terrestris* the highest (92% of the total number of root tips), and *Lactarius rufus* failing to form any mycorrhizas.

## 7. Domestication

In 1930, Sitka spruce seedlings were among those planted in British Columbia's first reforestation project in the Fraser River Valley, near Vancouver (Young, 1989). While previously planted at a level of about 10 million seedlings per year in British Columbia, Sitka spruce has been all but eliminated from reforestation programs in North America, due to damage from the white pine weevil (King *et al.*, 1998). Today, less than 2 million seedlings are planted each year, primarily on cool, coastal areas of the Queen Charlotte Islands. Meanwhile, Sitka spruce is the backbone of plantation forestry in Great Britain, accounting for about 70% of the seedlings planted (Malcolm, 1997), and is a commonly planted species in other European countries such as France and Denmark (Hermann, 1987). In spite of the good growth potential of Sitka spruce in the former Federal Republic of Germany, planting has been drastically reduced due to frost damage, drought and storm damage, and foraging by deer (Kleinschmidt, 1978).

### A. Deployment of reforestation materials

While Sitka spruce planting stock has traditionally been produced in bare root nurseries as 2+0 seedlings or 1+1 transplants, an increasing proportion is now produced in containerised growing systems, particularly in North America (Daniels and Simpson, 1990; Van Eerden and Gates, 1990; Aldhous and Mason, 1994). A variety of containers are used and stock is raised in both heated and unheated greenhouse structures. Cultural techniques have become highly sophisticated, ensuring that high-quality planting stock can be produced reliably and efficiently.

Sitka spruce planting stock can also be produced by means of vegetative propagation. Practical propagation systems have been developed (Kleinschmidt, 1992). Juvenile cuttings are easily rooted to produce planting stock as "stecklings" (Mason, 1984, 1992; John and Mason, 1987; Mason and Keenleyside, 1987) whose performance after planting is comparable to that of conventional transplant stock (Roulund, 1978; Roulund and Bergstedt, 1982; Baldwin and Mason, 1986; Mason *et al.*, 1989). While steckling planting stock has been actively promoted in Great Britain, higher nursery production costs have kept annual production to a few million (Mason, 1991, 1992; Mason and Sharpe, 1992). Despite the higher production costs for steckling stock, the ability to bulk-up (vegetatively multiply) scarce seed sources and tested crosses between selected individuals is expected to more than compensate by realising potential genetic gains earlier (Gill, 1983; Mason and Gill, 1986; Mason and Harper, 1987; Lee, 1992).

Techniques for the initiation and regeneration of somatic embryos are available (Krogstrup *et al.*, 1988), and embryogenic lines can be successfully regenerated after cryostorage (Find *et al.*, 1993; Kristensen *et al.*, 1994), making it possible to maintain genotypes in a completely juvenile condition during clonal testing. Sitka spruce plantlets derived from tissue culture propagation systems are also being automated further through the application of bioreactor technology (Moorhouse *et al.*, 1996).

Sitka spruce has been established by direct seeding on an experimental basis, where it has been shown that the seedlings produce a much smaller amount of adventitious roots, compared with bare root transplants (Coutts *et al.*, 1990). However, direct seeding has not been used operationally as a regeneration

technique for Sitka spruce. Its use in British Columbia is considered a poor option, due to the very slow growth of germinants, which predisposes them to drought and competition (Mitchell *et al.*, 1990).

## B. Provenance transfer

Within the native range, plantations established on sites with a strong maritime climate will be faster growing if seeds are transferred from more southerly latitudes. Conversely, transfers of seed from coastal origins to planting sites further inland involve higher risk (Lester *et al.*, 1990; Ying, 1990).

Sitka spruce seedlots from British Columbia have also been certified under the OECD scheme for sale in Europe (Pollard and Portlock, 1990; Portlock, 1996). In Britain, southern provenances (below 47°N latitude) grow fastest, but are susceptible to spring and autumn frosts (Lines, 1987a, b), although clonal testing has demonstrated substantial variation in frost hardiness within provenances and potential for selecting southern genotypes with low risk of frost damage (Nicoll *et al.*, 1996). Material from the Queen Charlotte Islands is generally recommended over much of Britain, although origins further south in Washington are better for south-west England, Wales, and parts of west Scotland (Fletcher, 1992). In the north of Germany, provenances from Washington are recommended, while fast-growing sources from Oregon are deemed to be too susceptible to frost damage (Stratmann and Tegeler, 1987). Provenances have been recommended for use in France, where plots have been established at four locations to demonstrate seed source differences (Bastien and Lemoine, 1986; François, 1986; Steinmetz, 1986). In Denmark, naturalised seed sources of the second and subsequent generations have grown faster and shown better adaptation than trees from seed imported directly from North America (Nielsen, 1994). In Germany, provenances from British Columbia (Canada) are also recommended.

## 8. Summary

Sitka spruce is an economically important species of the north temperate coastal rain forest of western North America. While not as commercially important as other conifers within its native range, it is a keystone species in some of the most productive ecosystems in North America. Sitka spruce is now widely planted in North Europe, where it forms the backbone of plantation forestry and is of enormous economic value in some regions. It is closely related to the other North American "white" spruces, *Picea glauca* and *P. engelmannii*. As an outcrossing, wind-pollinated species and prolific seed producer, it can transfer its genes rapidly to neighbouring populations and to other related spruces.

Sitka spruce exhibits clinal variation for many growth traits, associated with latitude, elevation, and distance from the coast. Population differences are also demonstrated for resistance to insect attack. While there is great variation among populations, more than half of the genetic variation in many growth traits is found among individuals within populations. Heritabilities for growth and quality traits are sufficiently high to expect substantial genetic gain from conventional recurrent-selection breeding programs. The species is readily propagated by rooted cuttings, offering potential to capture non-additive genetic variance and to accelerate the pace of genetic improvement. Statistically significant genotype-environment interactions have been observed, but broadly adapted individuals are rather common.

The distribution of Sitka spruce is limited to an area of maritime climate with abundant moisture. It may occur as pure stands, particularly on exposed coastal sites, but more commonly occurs in mixtures with western hemlock. While Sitka spruce is tolerant to shade and may occur in climax forest types, it is dependent on disturbance for regeneration and can be an aggressive pioneer in earlier stages of succession. The white pine weevil is by far the most serious threat to stands in North America, killing the leader and seriously affecting growth and merchantability. While the weevil does not affect planted stands in Europe, the green spruce aphid, various species of deer, and windthrow can cause significant damage.

Sitka spruce is well suited to artificial regeneration. While constituting a minor component of the reforestation effort within its native range, Sitka spruce plantation programs are well developed in some parts of Europe. Genetically improved materials from local seed orchards now constitute a significant portion of deployed planting stock. While most Sitka spruce reforestation is currently based on seed propagation, vegetative propagation techniques for cuttings and regeneration of somatic embryos are well advanced, making it a logical target for implementation of transgenic biotechnologies and the use of cloning in both breeding and deployment strategies.

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## **SECTION 6**

### **STONE FRUITS (*PRUNUS* SPP.)**

#### **I. Introduction**

##### **A. General Background**

The genus of *Prunus sensu lato* comprises more domesticated (also cultivated) species of temperate fruits than the other genera in the family of *Rosaceae* (*Malus*, *Pyrus*, *Sorbus*, *Cydonia*, *Rubus*, *Fragaria*). One of the obvious reasons for the abundant domestication might have been the coincidence between the location of the centre of variability of *Prunus* and the site of human evolution and/or of the first ancient high civilisations of human history.

Improvement of fruit trees through traditional breeding methods is a long-term effort because of their lengthy generation time. Thus, new approaches are researched to attain the envisaged breeding goals in a reasonable time frame. Genetic transformation is potentially useful, because specific genetic changes can be made. In the last few years successful examples of resistance breeding against viruses from different plant virus families have been reported, using the coat protein-mediated cross protection approach (Beachy *et al.*, 1990). However, only very few fruit trees have been among these experiments due to the difficulties in transformation protocols.

“Cross protection” was originally described as the phenomenon of protection of a plant against the invasion of a severe disease-causing virus due to prior inoculation of the plant with an attenuated virus strain (McKinney, 1929). Hamilton postulated in 1980 that the expression of sequences from the viral genome, if expressed in transgenic plants, could possibly cause a protection against viruses. In fact by the expression of the viral coat protein gene in transgenic plants, similar effects could be obtained, and it was therefore distinguished as coat protein mediated protection (Beachy *et al.*, 1990).

This still continues to be a difficult task among fruit trees, as can be seen from the limited number of successful reports of regeneration in woody species (McGranahan *et al.*, 1988; Laimer da Câmara Machado *et al.*, 1989; James and Dandekar, 1991; Oliveira and Pais, 1992; Mante *et al.*, 1991) and in *Prunus* species, in particular plum (Scorza *et al.*, 1994; Ravelonandro *et al.*, 1997) peach (Hammerschlag *et al.*, 1989), apricot and cherry rootstocks (Laimer da Câmara Machado *et al.*, 1992; da Câmara Machado *et al.*, 1995a,b).

##### **B. Topics for this case study**

One subject of the present study is to assess tools intended to cope with the new plagues, such as the currently incurable Plum Pox Virus (PPV) disease, which harass the growth of *Prunus* species and endanger the mere existence of the most precious, highly esteemed ancient, as well as, new cultivars.

Attempts at developing GMOs have been initiated by several research teams around the world and on such crops as plums (Scorza *et al.*, 1994) and apricots (Laimer da Câmara Machado *et al.*, 1992; da Câmara Machado *et al.*, 1995c). Similar research is currently sponsored in Hungary, especially, to save the traditional plum variety, Besztercei. This precious local variety found fame in the last century in Hungarian

and later Bosnian dried fruit, jam, distilled beverages, etc. The rapid decline of plum production ensued, as surmised, with the release of viruses triggered by the developing trade of nursery grown graftings. The plum growing regions of the former Yugoslavia were hit most severely. As a result, the original site of the variety Besztercei was assigned to Romania after the First World War. Traditional breeding to find or to introduce resistance to PPV started about the middle of the 20th century at Cacak (former Yugoslavia). In parallel, surveys of varieties resistant or at least less affected by PPV were undertaken, but, little hope exists of finding a solution (Cociu *et al.*, 1997; Hartmann, 1988).

In this study, an attempt is also made to provide background information for science-based decision making, in case such GMOs should be released into the environment. As well, this study focusses the assessment on risks and develops strategies for avoiding or counteracting said risks.

## 2. General Description and Use as a Crop

The genus *Prunus* is comprised of approximately 400 species of trees and shrubs. Many species and cultivars are grown for their edible fruits, while others are planted for their ornamental value. Approximately 25 species are native to the US, of which 18 reach tree size (Maynard *et al.*, 1991). Like *P. avium* L. in Europe, black cherry (*P. serotina* Ehrh.) is the only member of this genus with commercial importance as a timber species in the US (Fowells, 1965). It is a high-value hardwood timber species, prized for cabinetry, furniture and veneer (Panshin and De Zeeuw, 1970).

Stone fruits (Table 3.9) are appreciated world-wide either for fresh consumption, or in the processing industry: drying, distillation, canning, production of jams, syrups and fruit juices, etc. (Druart and Gruselle, 1986). As well, they are used for their timber and their value as ornamental crops (Moore and Ballington, 1991).

**Table 3.9 Stone fruit production (1000 metric tonnes) in the world from 1989 through 1999**

Crop	1989-91	1997	1999
Peaches and nectarines	9317	11286	12044
Plums	6270	7845	7346
Apricots	2226	2375	2720
Almonds	1288	1554	1632

Source : FAO Production Yearbook, 1999

Plum species are found native throughout the Northern Hemisphere but mostly in the temperate zone. The earliest writings about plums date back some 2,000 years (Gautier, 1977). Plums may have been the first species among all the fruits to attract human interest. It is more remarkable that the earliest cultivation of *P. domestica* began somewhere between Eastern Europe and the Caucasian mountains, whereas *P. salicina* and *P. simonii* were brought into cultivation in Asia.

It is remarkable that other cultivated temperate fruits of the *Prunus* genus, apricot and peach, reached Europe even before the Roman empire. The Latin names of the crops refer to Armenia and Persia, respectively, indicating the path of trade in ancient times. The centre of origin of those species is rather diffuse, but much more in the East, *i.e.* in Central and East Asia. Both species “grew up” as important crops in modern Europe. Some of the reasons might be their abundance and associated wealth as well as, a whole year round offer of subtropical fruits competing with the short season temperate fruits.

The peach is one of the most varied of all fruit species, falling between trees and shrubs of fruit. There are several types of them in the canopy, vegetative and generative characteristics, namely fruit, stone and

seed traits. All commercial cultivars belong to *P. persica* L. Batsch, and are primarily grown in temperate zones between latitudes 30° and 45° N and S, and in the tropics and subtropics at higher elevations (Hammerschlag, 1986).

A close relative of the peach, the almond, represents an entirely different food quality. Its cultivation in generally dry, if not marginal habitats, is as extensive as an almost semi-domesticated fruit, less subject to phytosanitary problems. Interspecific hybrids of *P. amygdalus* and *P. persica* are well known in fruit growing as an important rootstock for peach production, e.g., GF 677.

A somewhat detached subgenus of the genus *Prunus* includes the cherry and sour cherry which are, equally, ancient cultivated fruits, one of them being diploid, the other, tetraploid.

*P. avium* is primarily a European species, which occurs abundantly in wild form on the forest slopes of Southern, Central and Western Europe. Pomologically, according to fruit firmness, cherry cultivars are divided into the Heart cherry group, with mainly early ripening cultivars that have a soft flesh, and the Bigarreau group. The Bigarreau group includes late cultivars with firm flesh, such as Lambert, Stella, Bing, Van, Windsor, Schmidt, Hedelfingen, Napoleon, and Gold that have dark red, black, yellowish or light-coloured fruits. The major portion of the harvest is processed into solid, liquid or frozen products, and part is kept for direct consumption in the fresh state (Ivanicka and Pretová, 1986).

Sour cherry is widely naturalised and its distribution area covers almost all European countries and SW Asia. However, it is cultivated in many other parts of the world, mainly in North America. Sour cherry production is about one-third that of sweet cherry (FAO Yearbook, 1975, data not included in later editions). More recent data (in thousands tons) indicate that the most important producers are the USSR with 450 (Kramer, 1985) and the USA with 119 (Westwood, 1978). Other countries with great productions are: Germany (91), Former Yugoslavia (47), and Hungary (41) (Christensen, 1985; Kramer, 1985). Although the most important cultivars used are Schattenmorelle and Montmorency, the list of cultivars reaches a great number. Thus, in the USSR it runs up to 80 sour cherry varieties listed in various district catalogs (Kramer, 1985). The use of Stockton Morello in North America as a cherry rootstock is very minor (Tukey, 1964). The predominant root stocks in North America are mazzard (*Prunus avium*) or *P. mahaleb*.

The predominance of one or two major apricot cultivars in each production area is partly responsible for large fluctuations in yield and makes this crop species vulnerable to adverse environmental conditions, diseases and pests (Mehlenbacher *et al.*, 1991). Moreover, the major cultivars of the main apricot producing countries (Spain, Italy, the United States, Greece, France, Morocco, Hungary, Romania, South Africa, Bulgaria, Australia, Algeria) belong to the European group, which by their origin are known to have a very narrow genetic background (Kostina, 1969).

Apricot production is rapidly changing in Europe. Spain, the main producer keeps its production constant, while France is increasing production and Italy and Greece are decreasing their production levels.

Especially drastic is the situation in Greece, where the annual production of 100,000 tonnes about 10 years ago has decreased to 30-50,000 tonnes, mainly due to damage caused by late frosts and the Sharka virus.

The same holds true for Hungary, where at the beginning of the Seventies 60-130,000 tons were produced on an area of 13-14,000 ha, while in the early 90s the orchard area decreased to 2,500 – 3,000 ha and production dropped to 20-40,000 tons/a (Pedryc, Budapest, pers. comm).

### 3. Taxonomic Situation

#### A. Taxonomy

In the past different approaches were chosen to present the phylogeny of the subfamily of *Prunoideae* belonging to the family of *Rosaceae*. There were two main contrasting conceptions, *i.e.* all stone fruits belong to the genus *Prunus*, or the genus *Prunus* contains only plums and prunes. Here the classification is presented according to Strasburger *et al.* (1991).

The seven subgenera in *Prunus* are determined basically by how the leaves are rolled up in the bud, whether the flowers are organised in cymes or in racemes and finally by morphological characteristics of the generative organs, *i.e.* the size and colour of flowers, fruit, stone and seed traits.

- AMYGDALUS (almonds): **P. amygdalus**, *P. bucharica*, *P. fenzliana*, *P. kuramica*, *P. nana*, *P. orientalis*, *P. webbii*
- PERSICA (peaches): *P. davidiana*, *P. ferganensis*, *P. kansuensis*, *P. mira*, **P. persica**
- ARMENIACA (apricots): *P. ansu*, **P. armeniaca**, *P. brigantiaca*, *P. x dasycarpa*, *P. holosericea*, *P. mandshurica*, *P. mume*, *P. sibirica*
- PRUNUS (plums and prunes): *P. cerasifera*, *P. divaricata*, **P. domestica**, *P. insititia*, *P. italica*, *P. spinosa*, *P. syriaca*, **P. salicina**, *P. simonii*, *P. ussuriensis*, *P. americana*, *P. angustifolia*, *P. hortulana*, *P. maritima*, *P. mexicana*, *P. munsoniana*, *P. nigra*, *P. rivularis*, *P. subcordata*
- CERASUS (sweet and sour cherries): **P. avium**, **P. cerasus**, *P. fruticosa*, *P. japonica*, *P. maackii*, *P. mahaleb*, *P. pseudocerasus*, *P. pumila*, *P. serrulata*, *P. tomentosa*
- PADUS (bird cherries) *P. padus*, *P. serotina*
- LAUROCERASUS (bay-cherries)

This study will focus on essential data about the species in bold (*P. amygdalus*, *P. persica*, *P. armeniaca*, *P. domestica*, *P. avium*, *P. cerasus* and *P. salicina*), since they are the most widely grown species with horticultural interest. However, interactions with wild or escaped relatives will also be considered.

#### B. Number of chromosomes

The phenomenon of polyploidy is a widespread occurrence and of great importance in the evolution of new species or forms. For example, many genera of flowering plants contain a series of species characterised by varying degrees of ploidy. Polyploidy is important, too, from a practical point of view, since plants with this character are often very vigorous, and may be more resistant to frost and the attacks of parasitic fungi. Moreover, changes of flower structure and self-fertility according to the number of chromosomes have been observed. In *Prunus*, the basic number in vegetative cells is eight chromosomes. Polyploidy, due to interspecific hybridisation, took place during the phylogeny of the genus and is responsible for self-sterility and intersterility. The C-value is the DNA amount in the unreplicated haploid nucleus (pg/cell). The DNA amount in the unreplicated haploid or gametic nucleus of an organism is referred to as its C-value (Swift, 1950), irrespective of the ploidy level of the taxon. C-value equals genome size in diploid species, but always exceeds genome size in polyploid species. Nuclear DNA C-value and genome size are important biodiversity characters with fundamental biological significance and many uses (Bennett and Leitch, 1995).

The following different number of chromosomes and degrees of ploidy have been reported:

Genus	Species	Chromosome number	Reference
Amygdalus	<i>P. amygdalus</i>	2n=16 (diploid)	Darlington <i>et al.</i> , 1945
	<i>P. bucharica</i>	2n=16 (diploid)	
	<i>P. fenzliana</i>	2n=16 (diploid)	Darlington <i>et al.</i> , 1945
	<i>P. kuramica</i>	2n=16 (diploid)	
	<i>P. orientalis</i>	2n=16 (diploid)	
	<i>P. tenella</i>	2n=16 (diploid)	Darlington <i>et al.</i> , 1945

The cultivated almond was designated *Amygdalus communis* L. by Linnaeus in 1753. Miller (Webb, 1967) first used the name *Prunus* in 1768 in designating the cultivated 'sweet' almond as *Prunus dulcis*, describing it apparently as a 'botanical variety'. The species was later named *Prunus amygdalus* by Batsch (1801), the species name meaning 'Greek nut.' Archangeli (1882) later used the name *Prunus communis* for almond. Schneider (1904) and Rehder (1924) accepted *Prunus amygdalus* Batsch as the scientific name for almond and by which the species had been known in American botanical and horticultural literature for many years. In 1964, a discrepancy in name priority was determined to exist by the General Committee of Botanical Nomenclature of the International Botanical Congress (Punt, 1964). As a result, the name *Prunus dulcis* (Miller) D.A. Webb was proposed for the cultivated sweet almond (Webb, 1967). *Prunus amygdalus* Batsch (1801) and *Prunus communis* L. Archangeli (1882) are listed as synonyms. A flowering almond species appreciated as an ornamental is *Prunus triloba*.

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
<i>Persica</i>	<i>P. davidiana</i>		2n=16 (diploid)	Missouri Botanical Garden, 1990
	<i>P. ferganensis</i>		2n=16 (diploid)	Missouri Botanical Garden, 1991
	<i>P. kansuensis</i>		2n=16 (diploid)	Missouri Botanical Garden, 1990
	<i>P. mira</i>		2n=16 (diploid)	
	<i>P. persica</i>	0.6	2n=16 (diploid)	Darlington <i>et al.</i> , 1945 Bennett and Leitch, 1995

It has been suggested by Watkins (1979) that almond and peach, which are both regular diploids (2n = 16) originated from the same primitive species but evolved separately following the mountain development of the Central Asian massif. Almonds evolved in the arid steppes, deserts and mountainous areas to the west, south and southwest, whereas the peach evolved eastward towards China in a more humid environment and at lower elevations.

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
<i>Armeniaca</i>	<i>P. ansu</i>		2n=16 (diploid)	
	<i>P. armeniaca</i>	0.6	2n=16 (diploid)	Darlington <i>et al.</i> , 1945 Bennett and Leitch, 1995
	<i>P. brigantia</i>		2n=16 (diploid)	
	<i>P. mandshurica</i>		2n=16 (diploid)	
	<i>P. x dasycarpa</i>		2n=16 (diploid)	
	<i>P. holosericea</i>		2n=16 (diploid)	
	<i>P. mume</i>		2n=16 (diploid), 24	Darlington <i>et al.</i> , 1945
	<i>P. sibirica</i>		2n=16 (diploid)	

All apricot species are regular diploids with eight pairs of chromosomes ( $2n=16$ ). No difficulties have been reported in intercrossing *P. armeniaca*, *P. sibirica*, *P. mandshurica* and *P. mume*, although not all combinations have been attempted.

*P. x dasycarpa* Ehrh., the black or purple apricot, is a naturally occurring hybrid of *P. cerasifera* Ehrh. and *P. armeniaca* and is found as isolated trees, where the distribution of the two species overlaps (Mehlenbacher *et al.*, 1991). *P. x dasycarpa* has been backcrossed to both *P. cerasifera* and *P. armeniaca*; crosses to the plum parent are generally easier.

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
<i>Prunus</i>	<i>P. americana</i>		2n=16 (diploid)	Darlington <i>et al.</i> , 1945
	<i>P. angustifolia</i>		2n=16 (diploid)	Janick and Moore, 1975
	<i>P. cerasifera</i>		2n=16 (diploid), 24	Janick and Moore, 1975
	<i>P. domestica</i>	1.8	2n=48 (hexaploid)	Darlington <i>et al.</i> , 1945 Bennett and Leitch, 1995
	<i>P. hortulana</i>		2n=16 (diploid)	Janick and Moore, 1975
	<i>P. insititia</i>		2n=48 (hexaploid), 24	Darlington, 1945, Tischler, 1950
	<i>P. italica</i>		2n=48 (hexaploid)	
	<i>P. maritima</i>		2n=16 (diploid)	Darlington <i>et al.</i> , 1945
	<i>P. mexicana</i>		2n=16 (diploid)	Janick and Moore, 1975
	<i>P. munsoniana</i>		2n=16 (diploid)	Janick and Moore, 1975
	<i>P. nigra</i>		2n=16 (diploid)	Darlington <i>et al.</i> , 1945
	<i>P. rivularis</i>		2n=16 (diploid)	Janick and Moore, 1975
	<i>P. salicina</i>		2n=16 (diploid)	Darlington <i>et al.</i> , 1945
	<i>P. simonii</i>		2n=16 (diploid)	Janick and Moore, 1975
	<i>P. spinosa</i>		2n=32 (tetraploid), and natural hybrids with 16, 24, 40, 48	Darlington <i>et al.</i> , 1945, Janick and Moore, 1975
	<i>P. subcordata</i>		2n=16 (diploid)	Janick and Moore, 1975
	<i>P. salicina</i>		2n=16 (diploid)	Darlington <i>et al.</i> , 1945, Jannick and Moore, 1975
	<i>P. syriaca</i>		2n=16 (diploid)	
	<i>P. ussuriensis</i>		2n=16 (diploid)	

The most important commercial species of plums are generally classified in two groups, the European plums (*Prunus domestica* L.) and related forms with hexaploid chromosome number ( $2n=6x=48$ ) and the Japanese plums (*Prunus salicina*) and their hybrids with diploid chromosome number.

*P. domestica* is believed to have arisen as a natural allopolyploid between *Prunus cerasifera* (diploid) and *P. spinosa* (tetraploid) (Crane and Lawrence, 1952).

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
<i>Cerasus</i>	<i>Prunus avium</i>	0.7	2n=16 (diploid), 24, 32	Darlington <i>et al.</i> , 1945 Bennett and Leitch, 1995
	<i>P. besseyi</i>		2n=16 (diploid)	
	<i>P. cerasus</i>	1.2	2n=32 (tetraploid)	Missouri Botanical Garden, 1985 Bennett and Leitch, 1995
	<i>P. fruticosa</i>		2n=32 (tetraploid)	
	<i>P. mahaleb</i>		2n=16 (diploid)	Darlington <i>et al.</i> , 1945
	<i>P. pumila</i>		2n=16 (diploid)	Darlington <i>et al.</i> , 1945
	<i>P. serrulata</i>		2n=16 (diploid), 24	Darlington <i>et al.</i> , 1945
	<i>P. subhirtella</i>	0.6	2n=16 (diploid)	Darlington <i>et al.</i> , 1945 Bennett and Leitch, 1995
	<i>P. tomentosa</i>		2n=16 (diploid)	Darlington <i>et al.</i> , 1945

Among cherries, the sweet (*P. avium*) and sour (*P. cerasus*) cherry, flowering ornamental cherry species, and a few others used as rootstocks for cherries are important. The earliest description of the “keration” comes from Theophrastus about 300 B.C. *P. fruticosa*, the ground cherry, is considered the probable parent of both *P. avium* and *P. cerasus*, sweet and sour cherry respectively (Fogle, 1975).

The chromosome number of *P. cerasus* is 32 (Crane and Lawrence, 1952). As 8 is the base number of the genus *Prunus* and following De Candolle’s hypothesis (Coutanceau 1953) it seems that sour cherry is a tetraploid originating from an unreduced *P. avium* (2n = 16) gametophyte, thus by chromosome doubling.

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
<i>Padus</i>	<i>P. padus</i>		2n=16 (diploid)	Tischler, 1950
	<i>P. serotina</i>	1.0	2n=32 (tetraploid)	Kumar and Subramanian, 1987 Bennett and Leitch, 1995

### C. Molecular markers for the identification of genotypes

Since morphological markers sometimes are prone to equivocal interpretations and generally time consuming, the search for biochemical and molecular markers was initiated in the genus *Prunus*. The development of DNA markers like RFLPs or RAPDs is very recent in fruit trees (Eldredge *et al.*, 1992).

Initially, isoenzyme markers in *Prunus* crops like peach (Messeguer *et al.*, 1987; Monet and Gribault, 1991), almond (Cerezo *et al.*, 1989; Arús *et al.*, 1994a) and cherry (Santi and Lemoine, 1990; Boskovic and Tobutt, 1994) were developed.

In addition to the isozyme markers, RAPD, RFLP and AFLP (Arús *et al.*, 1994a,b) which are used to clearly distinguish among the different stone fruit cultivars available on the international fruit market, additional markers, such as SSRs have recently been developed for peach (Cipriani *et al.*, 1999) and apricot (da Camara Machado *et al.*, submitted).

#### 4. Centres of Origin/ Diversity

##### A. Geographic origin and natural distribution of *P. amygdalus*

Populations of almond have been described to occur in two areas: (a) the south west slopes and deep gorges of Kopet-Dagh Mountains of Turkmenistan (800-1700m), in an area, which is dry and snowless and (b) in Uzbekistan on the western slopes of the Tian Shan Mountains at similar elevations.

The almond *Prunus dulcis* (Miller) D.A Webb has been grown in cultivation for its edible seed since ancient times. From its centre of origin in Central Asia, it was disseminated to all ancient civilisation in Asia (2000 BC), Europe (350 B.C.), and North Africa (600-700 A.D.) (Vavilov, 1930; deCandolle, 1964). Almonds were initially introduced into California during the Spanish Mission Period, but significant plantings were not made until after the settlement of California following the Gold Rush (Wickson, 1910; Wood, 1925; Taylor and Philip, 1925). During the same period (1850-1900), almonds were introduced into West Australia (Quinn, 1928), South Africa, and parts of South America (particularly Chile and Argentina) in regions with the same climate as California.

Almond production is concentrated in three regions of the World: Asia, Mediterranean area (of Europe and Africa) and California (Kester and Horel, 1980).

The different species have different geographic distributions:

- *P. amygdalus* Batsch. syn. *P. communis* Arcang. is native in the south west slopes and deep gorges of the Kopet-Dagh and western slopes of the Tien-Shan Mountains
- *P. bucharica* (Korsh.) Fedtsch. is native in the steppes of Central Asia
- *P. feniziana* Fritsch. is distributed in the Caucasian Mountains, near Ararat and Armenia
- *P. kuramica* Korchinsky is feral native in slopes of the Hindukush Mountains
- *P. orientalis* Mill. is distributed in South-central Asia
- *P. webbii* (Spach) Vieh., a European almond species, which is found in the Balkans
- *P. tenella* is a European almond species with strong sprouting ability

##### B. Geographic origin and natural distribution of *P. persica*

Peaches are native to China and its culture dates back at least 4000 years (Wang, 1985). Wild peaches are known as “Maotao” (hairy peach) or “Yitao” (wild peach) currently exist in remote areas of China, where they are used as seedling rootstock for improved cultivars (Li, 1984). The Chinese recognise three groups of peaches (Li, 1984; Wang, 1985). The Southern group of peach is grown along the Yangtze River in the provinces of Jiangsu, Zhejiang, Jiangxi, Hubei, Hunan and Sichuan. The Northern group of peach is found along the Yellow River in Shandong, Hebei, Henan, Shanxi, Shaanxi, and Gansu provinces, and a third group is found in the arid northwest of China. Peaches spread west from China following the trade routes through Persia. In Egypt, peaches were used in offerings to the “God of Tranquillity” about 1400 B.C. (Roach, 1985). According to Plinius, the peach was planted in Greece by 332 B.C. and was mentioned by Virgil (70-19 B.C.) in Roman literature. Along the path of distribution through Europe, adapted populations of local peaches can be found. Among these are the “vineyard” peaches of France, Romania, and the former Yugoslavia (Parnia *et al.*, 1988) towards the Hungarian Great Plain. The peach was common in England by the 14<sup>th</sup> century (Bunyard, 1938). Peaches were brought to North and South America by the early Spanish explorers through St. Augustine, Florida and to settlements in Mexico by 1600.

Five species are considered as peaches:

- *P. davidiana* (Carr.) Franch. is native of North China
- *P. ferganensis* (Kost. et Rjyb.) Kov. et Kost., which is found in Western China
- *P. kansuensis* Rehd., which is native in North-western China
- *P. mira* Koehne, which is found in the Himalayan mountains and along the Yellow and Yangtze Rivers
- *P. persica* (L.) Batsch, which contains most of the cultivated peaches and nectarines (convarietas *leavis*), be it freestone (provar. *glabra*) or clingstone (provar. *nudicarpa*).

### C. Geographic origin and natural distribution of *P. armeniaca*

*Prunus armeniaca* L., the cultivated apricot, is believed to have originated in the mountains of Northern and North-eastern China in the same area as the Great Wall and overlapping the southern branch of the distribution of *P. sibirica* L. (Mehlenbacher *et al.*, 1991). The apricot was brought via Armenia and Asia Minor into Italy over 2000 years ago, to England in the 13<sup>th</sup> century and to North America only by 1720 (Westwood, 1978).

Wild apricots also occur in the Tien Shan Mountains in the Xinjiang autonomous region (Wang, 1985) and Dzhungar and Zailing Mountains in Soviet Central Asia. This is believed to be the secondary centre of origin (Zeven and de Wet, 1982). The area of distribution of the cultivated apricot is much larger and includes areas where seedling orchards are common such as Central Asia, Afghanistan, Kashmir, Iran, Turkey, and Trans-Caucasia (Kostina, 1936; Mehlenbacher *et al.*, 1991). All of these areas are valuable sources of germplasm.

Apricot production is severely restricted by ecological conditions. The gene pool of apricot contains only few species and varieties, which range in areas of adaptation from the cold winters of Siberia to the subtropical climate of North Africa and from California, the deserts of Central Asia and the humid areas of Japan and Eastern China. However, commercial production areas are still very limited (Mehlenbacher *et al.*, 1991; Faust - Surányi and Nyujtó, 1998).

In China, the Yu's order (2200 B.C.) refers to apricot growing and there are also documents from the 7th century (Löschner and Passeecker, 1954; Nyujtó and Surányi, 1981; Faust *et al.*, 1998). Kostina (1969) presented an excellent eco-geographical grouping for apricot cultivars and species. There are Central Asian, Irano-Caucasian, European, Northern Chinese, Tibetan, North-eastern Chinese, Eastern Chinese and Dshungar-Zailij groups (Faust *et al.*, 1998; Mehlenbacher *et al.*, 1991). Basic species are identified, as follows:

- *P. ansu* Maxim. is distributed in Eastern China, South Korea and Japan
- *P. armeniaca* L. is native of Northern and North-eastern China
- *P. brigantia* Vill. (Alpine apricot) is distributed in the region of the Alps, in South-eastern France
- *P. mandshurica* (Maxim.) Koehne is native in north east of China
- *P. x dasycarpa*, a hybrid between *P. cerasifera* and *P. armeniaca* Ehrh.
- *P. holosericea* (Batal) Kost. (Tibetan apricot), which is native of Tibetan Mountains
- *P. mume* (Sieb.) Sieb. et Zucc., which is native in south of China

- *P. sibirica* L., which is distributed along Baikal Lake, Mandshuria, North Korea

#### D. Geographic origin and natural distribution of *P. domestica* and *P. salicina*

Plum species are found native throughout the Northern Hemisphere but mostly in the temperate zone. The earliest writings about plums date back some 2000 years (Cullinan, 1937) and De Candolle assumes that plums have been known for 2000-4000 years (Banegal, 1954).

*Prunus domestica* seems to have originated in Southern Europe or Western Asia around the Caucasus Mountains and the Caspian sea (Cullinan, 1937). However, it is also widespread in the Balkans and Mediterranean countries.

*Prunus salicina* originated in China and was introduced into Japan 200-400 years ago. In China, it has been cultivated since ancient times where it is thought to occur in the wild in the Tsunglin range in Shensi and Kansu. Recently, it reached Europe by way of California and Italy.

Plums are a diverse group of plants with many botanical species, that have been cultivated for the last 3000 years. The most important species of *Prunus* are generally classified into three groups, the European, the Asian and the American plums. Plums may have been the first species among all the fruits to attract human interest. Six of the most important species of plums, *P. domestica*, *P. italica*, *P. syriaca*, *P. salicina*, *P. simonii* and *P. americana* are not known in the wild and presumably were selected and cultivated very early by humans. It is more remarkable that the earliest cultivation of *P. domestica* began somewhere between Eastern Europe and the Caucasian mountains, whereas *P. salicina* and *P. simonii* were brought into cultivation in Asia.

Regarding the Krieche/Haferpflaume (*P. insititia* var. *juliana*), repeatedly described as “wild”, Körber-Grohne (1996) mentions, that this is not the case in SW-Germany. As a welcome fruiting shrub, it has served as a shrubby hedge (Hag) around farm gardens or as a division between fruit orchards. It is not present in hedges or open fields nor is it found in woods or wood margins, as is the case with the crab apple. The oldest subfossil fruitstones have been found in Neolithic settlements in Germany and Switzerland (Ehrenstein, Robenhausen). The Krieche/Haferpflaume (*P. insititia* var. *juliana*) is a typical example of the continuity of domestication from the Neolithic until the present, which has been attributed to the propagation by grafting from Roman times onwards, or in the case of plum cultivars through root suckers.

The classification of plums is divided into geographic groups:

European group	<i>P. spinosa</i> L.	Europe, Asia Minor and North Africa
	<i>P. cerasifera</i> Ehrh. (and <i>P. divaricata</i> Ledeb.)	With some eco-geographical subspecies in Balkan, Asia Minor, Caucasian region and Central Asia
	<i>P. insititia</i> L.	In Central Europe, Balkan, Western Asia
	<i>P. domestica</i> L.	Native in Western Asia
	<i>P. italica</i> (Borkh.) em. Kárpáti	Hybrid between <i>P. domestica</i> and <i>P. insititia</i> with convarietas ( <i>pomariarum</i> , <i>claudiana</i> , <i>ovoidea</i> and <i>mamillaris</i> )
	<i>P. syriaca</i> (Borkh.) em. Kárpáti	Hybrid between <i>P. cerasifera</i> and <i>P. domestica</i>
Asian group	<i>P. salicina</i>	Native in the Basin of Yangtze River
	<i>P. simonii</i>	No wild form, only cultivated
	<i>P. ussuriensis</i>	Along Ussuri River
American group	<i>P. subcordata</i>	Native of California and Oregon
	<i>P. mexicana</i>	South-western Kentucky to Western Tennessee to Oklahoma and Mexico
	<i>P. rivularis</i>	Native in Texas
	<i>P. maritima</i>	From Brunswick to Virginia
	<i>P. americana</i>	From Massachusetts to Georgia to near the Gulf of Mexico and to the west
	<i>P. nigra</i>	From New Brunswick to Northern Ohio
	<i>P. angustifolia</i>	From Delaware to Florida and Texas
	<i>P. hortulana</i>	Native in Central Kentucky and Tennessee, to Iowa and Oklahoma
	<i>P. munsoniana</i>	From Kentucky to Kansas and Texas

### E. Geographic origin and natural distribution of *P. avium* and *P. cerasus*

Watkins (1976) suggests that the first diploid *Prunus* species arose in central Asia, and that species in the section *Cerasus* which includes sweet, sour and ground cherry, were early derivatives of this ancestral *Prunus*. The *Cerasus* cherries developed to the west of the central Asian subgenus *Cerasus*' centre of origin while most other *Cerasus* species evolved to the east.

Hedrick (1915) described the geographic range of wild sweet cherry as all of mainland Europe well into the Southern U.S.S.R. and as far east as Northern India, with the greatest prevalence between the Caspian sea and the Black sea. In contrast, the ground cherry centre of origin is Western and Central Asia (Watkins, 1976).

It is reported that sweet cherries (*Prunus avium*) were brought in 74 B.C by the Roman General Lucullus from Cerasunt on the Black Sea to Rome, and from there spread to Germany and Britain.

Sweet cherries (mazzard) have been grown from Southern Russia, north of the Caucasian mountains to the north of France for a long time. *Prunus fruticosa* Pall., the ground cherry, has a wider area of distribution, which overlaps with the centre of wild cherry, thus giving new hybrids as sour cherries. The sour cherry is native in the Carpathian Basin. Domestication and cultivation has resulted in some ecotypes of sweet and sour, or ground cherries in the various areas of Europe, and partly in Asia. Several sweet and sour types are adapted to harsh winter conditions (Iezzoni *et al.*, 1990; Faust and Surányi, 1997).

Although cultivated cherries are ubiquitous in the temperate zone, there has been little effort to take them further south into subtropical regions. There are those cultivars which require low-chilling among the cherry genotypes, although the existing high quality cherry cultivars all have high chilling requirements. Commercial production of sweet cherries is limited by rain fall during the ripening period, which causes cracking of Bigarreau-type sweet cherries and subsequent brown-rot infection, which destroys the value of the fruit.

Sour cherries are not subject to cracking, nevertheless, they are also better adapted to cool climates. Most of the world cherry production is in Europe, its native home.

Duke cherries are hybrids between sweet and sour cherry varieties.

Wild and cultivated species are useful in breeding and cultivation:

<i>P. avium</i> L.	native of Eurasia
<i>P. cerasus</i> L.	a hybrid between <i>P. avium</i> and <i>P. fruticosa</i>
<i>P. fruticosa</i> Pall.	grows in Southeast Asia and Central and Western Europe
<i>P. mahaleb</i> L.	grows in Southern and Central Europe and Asia Minor
<i>P. pseudocerasus</i> L.	originated in the North China
<i>P. tomentosa</i> Thunb.	native in the Chinese provinces and Eastern Tibet

Furthermore, there exist American and Asian ecotypes:

- *P. capuli* L. Mexican cherry
- *P. japonica* Thunb.
- *P. maackii* Rupr.
- *P. pumila* L.
- *P. serrulata* Lindl.

## 5. Reproduction Biology of the Genus *Prunus*

### A. Sexual reproduction

This genus is exceptional in the *Rosaceae* family - representing the evolutionally most advanced taxon - with a pistil reduced to one carpel only. In the pistil, there are regularly two ovules but in the majority of cases only one grows to seed. The rate of two (or even more) seeds per stone are sometimes typical for particular varieties in Pomaceous pistils but are rare in *Prunus*. The androeceum, with its three whorls of stamens with regular anthers does not show much difference from the average of the family. Seeds of pollinated fruits with embryos issued from zygotes as a product of fused sexual cells (gametes) are the main form of reproduction, even though interspecific hybrids with generative sterility and many cultivars are able to produce tillers. There is no indication of apogamy or parthenogenesis, neither of parthenocarp, except in some varieties with signs of aborted embryos as a result of selection for extra early ripening. In that case, however, *in vitro* rescue of excised embryos may secure the survival of the offspring. The genetic dynamics of the species are secured by a high rate of outbreeding conditioned by a polyallelic system of autoincompatibility (2.4.2). The seeds are tightly closed in the stone, *i.e.*, the lignified endocarp of the fruit. For germination, however, the seeds need to be stratified, *i.e.*, exposed to temperatures below 10°C for some weeks or months, practically over the winter to start germination, moreover, excised seedlings from ripe seeds also need some “chilling” in order to develop normally, *i.e.* to produce elongated shoots axes. The same holds true for the excised, underdeveloped embryos rescued for breeding purposes.

In almond, self-incompatibility is controlled by different alleles of a gametophytic self incompatibility gene (Kester and Asay, 1975; Crossa-Raynaud and Grasselly, 1985; Socias i Company and Felipe, 1988). Self-fertility genes have been found in almond and related species. Recently, 17 additional self-fertile genotypes have been identified in wild almond populations in Italy (Reina *et al.*, 1985).

*Prunus persica* is self-fertile. However, pollen sterility in *P. persica* could be useful for interspecific hybridisation.

In apricot, most Central Asian cultivars are self-incompatible, while most European cultivars are self-compatible (Mehlenbacher *et al.*, 1991).

Most sweet cherry cultivars presently being grown are self-incompatible. Self-incompatibility in sweet cherry was first identified by East and Mangelsdorf (1925) and later determined to be of the monofactorial gametophytic type with multiple allelic S locus (Lewis, 1948).

Self-fertility in sweet cherry has been obtained through X-ray radiation applied to flower buds at the pollen mother cell stage (Lewis, 1948; Lewis and Crowe, 1954) and by spontaneously occurring mutations (Lewis, 1951).

### **B. The mating system of most cultivated *Prunus* species**

The mating system of most cultivated *Prunus* species is determined by the clearly defined system of auto-incompatibility, which is inherently combined with inter-incompatibility. However, the high number (20-40) of known alleles of the single (Sx) locus with gametophytic determination allows little chance of meeting incompatible mates amongst the varieties cultivated. Those incompatible combinations are registered according to experimental proofs accumulated during the last, nearly seventy years since the phenomenon has been detected. Auto-incompatibility is expressed at different degrees between total, intermediate and scarcely identified cases. The apparent lack of such a mechanism is found in peach, although male sterility occurs at a relatively high frequency, *e.g.*, the cultivar J.H. Hale, and in some plums, *e.g.*, the cvs Tuleu gras, Pitestean and Carpentin (Silbereisen *et al.*, 1996). The evolutionary role of male sterility is highly analogous to that of incompatibility. As a general tendency of the domestication process, the increased rate of self-fertility is derived from the selection pressure for high and regular yields in crops grown for their generative organs. This can be seen in the *Prunus* species as many, mainly recently developed, varieties are self-fertile. In modern times, self-fertility has been consciously favoured. Another condition of self-fertility is polyploidy appearing on the margin of the spreading species like the European plum and sour cherry as amphiploids of interspecific hybrids in which activity of the S alleles is impaired. No doubt that some of those clones are also tillering spontaneously. Conditions of cultivation, introduction to new habitats included, are similar to the marginal areas of a species where rare mutations enjoy better chances to survive. All those reasons enhance the chances of self-fertile genotypes. Some exceptions in European plum and sour cherry prove that auto-incompatibility did not disappear entirely with amphiploidy, so a conscious effort in the research for high productivity gave rise to new, self-fertile sour cherry cultivars.

Search for radiation-induced mutants in cherry populations has spotted the Sf allele, which proved to be dominant in relation to the rest of the sterility alleles. This phenomena is employed in breeding programmes in order to produce new self-fertile cherry cultivars. The first self-fertile sweet cherry cultivar was released from a breeding program in 1968 and named Stella (Lapins, 1971). It was the result of a cross between Lambert and JI 2420. The cross was made in 1956. JI 2420 came from the John Innes Institute in the UK and was the result of a cross between Emperor Francis and irradiated pollen from Napoleon. At the moment all self-fertile cultivars have Stella in its pedigree somewhere. The John Innes Institute had a couple of other selections that were self-fertile but they have not yet made it into a named cultivar.

Bees play a major role as pollinating agents, as the pollen of *Prunus* species cannot be carried by wind and even self-pollination requires the mechanical intervention of insects.

### C. Natural vegetative multiplication

Natural vegetative multiplication is rather exceptional in the genus of *Prunus*, but all subgenera have members which build up extended colonies by tillering, and some successful cultivars have been maintained by tillers since ancient times, e.g., European (hexaploid) plums in the NE-Hungary (in the riverside of the Tisza river) and there are clones of semi-wild cultigens, blackthorn and sour cherry. Most of the existing cultivars are, however, multiplied by grafting and trading, except, some old varieties, such as the sour cherry variety Cigánymeggy (Hungary) and Oblacinska (Former Yugoslavia). In the group of almonds the only tillering wild species, *P. tenella*, is eligible as a potentially dwarfing stock.

More attempts have been invested into the vegetative propagation of *Prunus* rootstocks. As very few are inclined to develop tillers spontaneously, the tools of micropropagation *in vitro* gained particularly high interest, for example, in the rapid multiplication of GF 677. In fact, millions of plants are produced worldwide by *in vitro* techniques (Rosati and de Paoli, 1992). The production of self-rooted plantlets by green cuttings under a mist curtain seems to be less favoured mainly because of the questionable value of the self-rooted trees and/or the low efficiency of the technique. Grafting techniques, on the other hand, became routine several centuries ago. In addition, some stocks are propagated with cuttings.

## 6. Crossability

### A. Interspecific and intergeneric hybrids

Interspecific (and intergeneric) relations in *Prunus* are not clear, perhaps due to the widespread presence of auto-incompatibility and the relative fertility of interspecific hybrids. The prolonged time period for flowering in the *Prunus* species and even within varieties of one species, substantially influences the possibility of mutual pollination between different cultivars, as well as different species. This is due to the different phases in flowering during the blooming season of the cultivated *Prunus* species.

The physiological or ecological diversity of the species caused sufficient isolation of their individual habitat. Consequently, they were scarcely sympatric in their natural environment, whereas, some *Prunus* species were grown, regularly, in home gardens if not in larger orchards. Interspecific barriers did not develop during natural evolution, but by the appearance of cultigenous hybrids, which triggered their development from the first steps of domestication up to the limits set by taxonomic divergence. Although the cherries are perhaps the most distant from the rest of the species, we find bridging species between plums and cherries as documented for *P. salicina*. Less difficult seems to be the gene flow between plum-apricot-peach and almond as documented by the list of successful interspecific crosses (Table 3.10).

Table 3.10 Interspecific hybrids with *Prunus persica*

<i>P. amygdalus</i>	x <i>P. persica</i>
	x ( <i>P. amygdalus</i> x <i>P. davidiana</i> )
<i>P. armeniaca</i>	x <i>P. davidiana</i>
	x <i>P. persica</i>
<i>P. besseyi</i>	x <i>P. persica</i>
<i>P. cerasus</i>	x <i>P. persica</i>
<i>P. hortulana</i>	x <i>P. persica</i>
<i>P. nana</i>	x <i>P. persica</i>
<i>P. persica</i>	x <i>P. amygdalus</i>
	x <i>P. davidiana</i>
	x <i>P. cerasifera</i> var. <i>divaricata</i>
	x cherry (sps?)
	x <i>P. kansuensis</i>
	x <i>P. mira</i>
	x <i>P. nana</i>
	x <i>P. besseyi</i>
	x <i>P. salicina</i>
	x <i>P. spinosa</i>
<i>P. salicina</i>	x <i>P. persica</i>
<i>P. spinosa</i>	x <i>P. persica</i>
<i>P. tenella</i>	x <i>P. davidiana</i>
	x <i>P. persica</i>

Source : after Janick and Moore, 1975

All the species mentioned have been intercrossed with various degrees of difficulty, and grafted on each other within reasonable limits, which is proof of their genetic and physiological affinities. The use of rootstocks enlarges, dramatically, the possibilities of occupying ecological niches previously inaccessible with species on their own roots.

Hybridisation readily takes place between *Prunus amygdalus* and *Prunus persica* (Kester and Asay, 1975, 1988). Naturally interspecific hybrid rootstocks, 'GF 667', are common, where the two species are grown together.

North-American species and their interspecific hybrids, created between 1907 and 1965, represent a distinct group of cultivated *Prunus* species, the "cherry plums" (different from the species *P. cerasifera*, the cherry plum in the traditional sense). They are derived essentially from *P. besseyi* and *P. pumila*, with the western and the eastern sand cherry as a common parent (Janick and Moore, 1975) (Table 3.11).

**Table 3.11 Hybrids of the sand cherries (*P. besseyi* and *P. pumila*) with other species**

Sand Cherry	x <i>P. americana</i>
Sand Cherry	x <i>P. salicina</i>
Sand Cherry	x <i>P. salicina</i>
Sand Cherry	x <i>P. simoni</i>
Sand Cherry	x <i>P. armeniaca</i>
Sand Cherry	x <i>P. persica</i>

Source : Janick and Moore, 1975)

It is remarkable that peach (*P. persica*) is one of the most flexible species of *Prunus* regarding its use in interspecific crosses for breeding purposes. The documented products of these breeding efforts are divided into two distinct groups: Hybrids mostly fertile (A) and mostly sterile (B). A complicated polyhybrid background is surmised in most cases from taxa *P. davidiana* (d), *ferganensis* (f), *kansuensis* (k), *mira* (m) and *persica* (p) according to Scorza and Okie (1990) and Janick and Moore (1996) (Table 3.12). In group (A), we may consider the hybrid products as potentially new fruits, *i.e.* distinct commodities, as it happened with the sand cherry derivatives in the Midwest of the United States.

Table 3.12 *Prunus* species reported as hybrids between peach and peach species

Species		Hybrid	Common name	Origin
<i>P. amygdalus</i>	(A)	d.m.p.	almond	SW Asia
<i>P. davidiana</i>	(A)	k.p.	mountain peach, shan tao	N China
<i>P. ferganensis</i>	(A)	p.	xinjiang tao	NE China, S Russia
<i>P. kansuensis</i>	(A)	d.p.	wild peach, kansu tao	NW China
<i>P. mira</i>	(A)	p.	Tibetan peach, xizang tao, smooth-pit	W China-Himalayas
<i>P. persica</i>	(A)	d.f.k.m.	peach, maotao	China
<i>P. americana</i>	(B)	p.	American plum	USA
<i>P. armeniaca</i>	(B)	d.p.	Apricot	Asia
<i>P. besseyi</i>	(B)	d.p.	western sand cherry	N USA, Canada
<i>P. brigantina</i>	(B)	p.	Briancon apricot	France
<i>P. cerasifera</i>	(B)	d.p.	myrabolan plum	W Asia
<i>P. cerasus</i>	(B)	p.	sour cherry	W Asia, SE Europe
<i>P. domestica</i>	(B)	p.	European plum	W Asia, Europe
<i>P. hortulana</i>	(B)	p.	wild plum	C USA
<i>P. japonica</i>	(B)	p.	Chinese bush cherry, Korean b.c.	China
Species		Hybrid	Common name	Origin
<i>P. nigra</i>	(B)	p.	Canadian plum	N USA, Canada
<i>P. pumila</i>	(B)	p.	eastern sand cherry	N USA
<i>P. salicina</i>	(B)	f.p.	Japanese plum	China
<i>P. simmonii</i>	(B)	p.	Simon's plum	N China
<i>P. spinosa</i>	(B)	p.	sloe or blackthorn	Europe, W Asia, N Africa
<i>P. tenella</i> = <i>nana</i>	(B)	d.p.	Siberian almond	SE Europe, W Asia
<i>P. tomentosa</i>	(B)	p.	Chinese bush cherry, Manchu cherry	N&W China, Japan
<i>P. virginiana</i>	(B)	p.	choke cherry	N USA, Canada

(A) Closely related to peach producing fertile hybrids

(B) Hybrids mostly sterile. The codes of species used as parent in the ancestry:

*P. davidiana* (d), *ferganensis* (f), *kansuensis* (k), *mira* (m) and *persica* (p)

The only valid example of commercially recommended interspecific pollinations is between sweet and sour cherry (Nyéki and Soltész, 1996). As parthenocarp, understood as seedless fruit, does not exist in *Prunus*, it seems obvious that seed abortion might have little chance in fruit production, however, some extra early ripening cherry and peach varieties are used to develop unviable seeds. It was proved that the excision of the embryos before the fruit have ripened facilitates the rescue of plantlets under *in vitro* conditions. Thus an efficacious technique has been developed for use by breeders in combining genes of those extra early varieties, e.g., Bailey and Hough.

Because of the ease of natural hybridisation of *P. fruticosa* with *P. cerasus* and *P. avium*, some *P. fruticosa* rootstocks under testing may be interspecific hybrids.

*Prunus tomentosa* has been hybridised with cherry (Fisher and Schmidt, 1938; Noznikov, 1951). *Prunus salicina* hybridises easily with *P. simonii*, *P. armeniaca*, and American plum species.

No difficulties have been reported in intercrossing *P. armeniaca*, *P. sibirica*, *P. mandshurica* and *P. mume*, although not all combinations have been attempted. *P. x dasycarpa* has been backcrossed to both *P. cerasifera* and *P. armeniaca*; crosses to the plum parent are generally easier.

Results to date indicate that crosses between true apricot species (*P. armeniaca*, *P. mandshurica*, *P. sibirica* and *P. mume*) are successful when made in either direction and resulting hybrids are viable and fertile.

A large number of crosses between various plum and apricot species have been reported. Listed in order of flowering date, they are *P. salicina* Lindl., *P. x dasycarpa*, *P. cerasifera* Ehrh., *P. domestica* L., *P. besseyi* Bailey, and *P. maritima* Marsh. The initial cross is generally more successful when plums are used as the female parent. *P. cerasifera* x *P. armeniaca* produced hybrids resembling the natural interspecific hybrid species *P. x dasycarpa*.

Hybrids of the Asian plum species *P. salicina* with *P. armeniaca* have also been generated with little difficulty. Fertility of the hybrids varies; pollen fertility is generally quite low.

Several authors also report successful hybridisation of the hexaploid plum *P. domestica* with apricot. Resulting hybrids are tetraploid.

The beach plum, *P. maritima* has also been hybridised successfully with common apricot. More distant hybrids of apricots with peach and almond have been reported. These crosses are quite difficult to make and the resulting hybrids are often weak and sterile. The incorporation of genes from *P. persica* could conceivably greatly expand the areas in which apricots could be grown.

## B. Introgression into wild relatives

The introgression between cultivated and wild species is scarcely documented. There is no doubt concerning the physical possibility. Escapes of cultivated varieties are frequently found in woods, pastures, abandoned orchards, ruderal, suburban, and marginal areas. Intercrosses with really wild populations have very little chance, as blackthorn, hedge cherry and dwarf almond (*P. tenella*) are extremely different in morphology, as well as in adaptation, *i.e.* eventual hybrids could only survive in a much protected environment. Cherries may have more chances as far as introgression into the wild populations is concerned. It is worthwhile to consider the escapes of varieties and species introduced as rootstocks to nurseries and grown out from the roots and stumps of destroyed grafts in abandoned orchards. That is how a high diversity of cherry plums have been naturalised recently. As a result, the cherry plum has become much more tolerant than the European plum and apricot to the destructive effect of Plum Pox Virus (PPV). Escaped rootstock varieties and spontaneous hybrids of ancient, as well as, recently introduced varieties are a general phenomena found in neglected orchards, and escapes of no immediate relation to fruits growing in the area are found. For example, *P. serotina*, *P. mahaleb*, *P. padus*, bitter almonds.

In Central Europe, the possibility of introgression is much more limited to the Near East, Caucasus, Iran, Central Asia and the Chinese subcontinent, where a huge wealth of intermediate and semi-cultivated forms reside.

## 7. Domestication of *Prunus* sp.

### A. Breeding of *Prunus*

One of the obvious reasons for the abundant domestication of the *Prunus* species might have been the coincidence between the centre of variability of *Prunus* and the site of human evolution and/or of the first ancient high civilisations of human history. The easily fossilised stone of the fruit proved that fruit of

considerable size existed long before the appearance of man. Plums “offered themselves” to man to be domesticated. According to ecological arguments, it is highly probable that the today despised species, e.g., blackthorn (*P. spinosa*) followed men as a “secondary crop” which is reflected in its occurrence as a witness of the ancient Neolithic culture on the outskirts of the villages, roadsides and pastures as hedges. The ancestors of that tetraploid species are unknown. It survived adversities of severe pasturage but benefited from deforestation during the spreading of primitive agriculture in the Near East and Europe, where its more vigorous relative, the favoured fruit tree, the diploid cherry plum (*P. cerasifera*) presented a permanent temptation for crossbreeding. The appearance of the European plum (*P. domestica* and *insititia* included) was not an unique and endemic event in the history of the Eurasian region. The hexaploid, (amphiploid) species has been reproduced at several instances by purposeful breeding, according to the model of bread wheat, triticale, tobacco, oilseed rape, garden strawberry, and other cultivated species, first perhaps by Rybin, a disciple of Vavilov in the 1930s. Since ancient and medieval times, the European plum made an important carrier, first owing to its ability of producing tillers as its alleged ancestor, the blackthorn also did, and secondly, because it became naturalised in mesophytic marginal cultivated areas and some river flats (e.g., of Felső-Tisza). As the most important fruit and almost staple food, it served the well being of poor people for centuries. However, later on, it became one of the first horticultural products to be exported and became of this, was seen as a symbol of wealth (as the greater number of plum trees on the manor of a member of the gentry, the more wealthy he was). The distilled drink achieved its fame as the national drink in S-E Europe, replacing gin. Recently, the fate of the European plum has been severely impaired, by the Plum Pox Virus, which was identified during the first part of the 20th century.

In the rich choice of plums, a host of species originating from East Asia and North America are diploid. These species have been inter-crossed with the cherry plum with considerable success. As a result, in the last few decades there has been an impetus of the so-called Japanese Plums. The first documented attempts are due to Luther Burbank, a Californian breeder at the end of the 19th century. Those interspecific hybrids, however, well represented in the list of cultivars, only represent a small fragment of the huge gene reserves of the Northern Hemisphere. As a source of precious genes, especially for resistance and special qualities lacking from the traditional European plum, these varieties are to be kept in mind.

## **B. Conservation of *Prunus* genetic resources**

The International Plant Genetic Resources Institute (IPGRI), formerly known as the International Board for Plant Genetic Resources (IBPGR) has elaborated a descriptor list for plum, peach, cherry and apricot (IBPGR 1984a,b,c, 1995) and developed recommendations for the safe movement of germplasm of stone fruits (Diekmann and Putter, 1996).

The European Information Platform on Crop Genetic Resources has been established under the umbrella of the European Co-operative Programme for Crop Genetic Resources Networks (ECP/GR) to facilitate access to information about genetic resources conserved in genebanks throughout the Region. The European *Prunus* database has been maintained by Dr. Anne Zanetto at the “Institut National de la Recherche Agronomique” (INRA) in Bordeaux, France, under the initiative of the European Co-operative Programme for Crop Genetic Resources Networks (ECP/GR) since 1994. The database includes, in 26 European countries, the collections of all *Prunus* species, cultivated stone fruit and their related species (even the wild ones). The database is comprised of 19 passport data from the IPGRI/FAO Multicrop descriptors list, 13 descriptors common for all the different species and 3 to 7 specific descriptors depending on the species of the accession. These descriptors are mainly morphological. The possibility of including more agronomic or physiological descriptors is under consideration by the ECP/GR *Prunus* Working Group. The database has been supported for three years by the European Union in the “European Programme on the conservation, characterisation, collection and utilisation of genetic resources in

agriculture”, under the title “International Network on *Prunus* genetic resources” (GENRES61). In addition, there are gene banks in China and Japan.

### C. Synecology

In Europe, some wild, native and escape species of *P. nana*, *P. avium*, *P. fruticosa*, *P. mahaleb* and *P. spinosa* can be found in natural and cultivated forests or ruderals.

### D. Interaction with pathogens

*Rosaceae*, in general, and *Prunus* species, in particular, are prone, in varying degrees, to infections by a range of pathogens, e.g. fungi such as *Monilia laxa*, *Taphrina deformans*, bacteria, such as *Pseudomonas*, and *Xanthomonas*, viruses, such as PPV, PNRSV and PDV and phytoplasmas such as European Stone Fruit Yellows (ESFY) (<http://www.boku.ac.at/pbiotech/phytopath>).

The main problems associated with apricot growing include: die-back or apoplexy, sensitivity to viruses, frost damage in winter and spring, fungi cankers and alternate bearing.

In recent years, a viral pathogen became the major threat to stone fruit cultivations in large areas of Central and Southern Europe and other Mediterranean countries. This pathogen was responsible for considerable economic losses and reduction of production areas. The Plum Pox Virus (PPV), causal agent of Sharka disease and a member of the potyvirus family, was classified by US and EC plant quarantine agencies as the most important pathogen in apricots, plums and peaches (the only plant pathogen for which an APHIS plan exists (Scorza, 1991). Sharka infection data from Spain, Greece, France and Italy clearly demonstrates the economic consequences of this threat (COST 88 Plum Pox Virus Workshop -Potyvirus Group, Valencia June 1993), as apricot cultures are increasingly being replaced. Apricot appears to be the most sensitive stone fruit towards infection with PPV. Apricot production was practically erased in the late 70s in some valleys of Northern Italy, e.g., Vintschgau (Eynard *et al.*, 1991), and is seriously threatened in some Austrian valleys, e.g. Wachau (Pieber, pers. comm). After its appearance in the South American Continent (Herrera *et al.*, 1997) in 1999, it has been confirmed for the first time in the US (<http://aphis.usda.gov/lpa/press/1999/10/plumpox.txt>). This prompted Canada to close the entry of *Prunus* material from the US (<http://www.cfia-acia.agr.ca/english/corpafr/newsrelease/19991122e.shtml>).

Considering the severity of the disease, the difficulty to control its spread, and the lack of resistant cultivars, the necessity of resistant cultivars is evident and a straight-forward strategy is required. In fact there are no species resistant to Plum Pox Virus among the species sexually compatible to crop cultivars. This means it is not possible to obtain resistant cultivars by conventional hybridisation.

The information concerning the interrelation of *Prunus* species gives an opportunity of considering their pathosystems. Most pathogens and pests have developed along evolutionary pathways in parallel with the domestication and evolution of new species and hybrids. Because of geographical continuity, most species coming from the East carried their parasites freely. One of the best examples is the green peach aphid, *Myzus persicae*, which proved its fidelity to the peach tree in spite of being polyphagous; neither related species substituted the peach as a primary host. It is remarkable that even though the peach was one of the last oriental species to arrive in Europe, the green peach aphid became one of the most efficient vectors of viral diseases in Northern Europe. On the contrary, a pathogen *Taphrina deformans* did not harm either of the relatives of peach. Although, another adapted disease, *Polystigma rubrum* kept to the European plum (and blackthorn) as host, in spite of the permanent sympatric presence of relatives of the *Prunus* genus. Different susceptibilities to other diseases and pests indicates the existence of genetic resistance in spite of the possibly, small genetic divergence, e.g. the reduced proliferation of aphids on apricots in relation to peach and plum.

The increasing threat of stone fruit production in Mediterranean countries by phytoplasma diseases has been recognised in recent years. Although severe decline of European stone fruits was reported as early as 1924 on apricots in France and in 1933 on Japanese plums, in Italy it was only in 1973 that their phytoplasma aetiology was discovered. At that time, phytoplasmas were called mycoplasma-like organisms (MLOs). As different *Prunus* species were affected, different disease names were given: apricot chlorotic leaf roll (ACLR) on apricots, plum leptonecrosis (PLN) on Japanese plums, peach yellows, peach rosette and peach vein clearing (PVC) on peach and several other decline diseases on European plum, almond and flowering cherry. *Prunus* rootstocks are also severely affected by similar disorders. Common symptoms are yellowing and leaf roll in summer, off-season growth in winter, die-back and a more or less rapid decline. Up to now these diseases have been restricted to the southern half of Europe with their northern border in Germany. In the past few decades they have been of increasing economic importance, e.g. ACLR and PLN are especially devastating for apricots and Japanese plums. Molecular analysis of the pathogen revealed that only one type of phytoplasma, the European stone fruit yellows (ESFY) phytoplasma, is associated with all these diseases (Jarausch *et al.*, 2000). ESFY phytoplasmas are genetically different from phytoplasmas infecting *Prunus* species in North America. ESFY phytoplasmas are classified as quarantine organisms by European legislation (Laimer da Câmara Machado *et al.*, 2001; Heinrich *et al.*, 2001). Upon experimental inoculation, apricot, peach and Japanese plum are the most susceptible stone fruits whereas European plum and almond are more tolerant and cherries appear to be resistant (Jarausch *et al.*, 2000).

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## SECTION 7 WHITE SPRUCE (*PICEA GLAUCA* (MOENCH) VOSS)

### 1. Forestry Practices

White Spruce is the most commonly planted tree species in Canada, accounting for more than one-third of all reforestation throughout the (Kuhnke 1989). Active tree breeding and orchard programmes exist in every Canadian province, a distinction not shared by any other species (Fowler and Morgenstern 1990; Lavereau 1995). Breeding programmes in the United States are active, with productive White Spruce seed orchards in New England, New York and the Lake States (Carter and Simpson 1985; Carter 1988; Stine *et al.* 1995). ). White Spruce is the dominant reforestation species in the upper Lake States (Rauscher 1987). In Germany, *Picea glauca* has had limited use as a species with potential for reclaiming badly polluted industrial areas (Weiss 1986). Elsewhere in the world, White Spruce is of much less importance. With the exception of limited ornamental use, it is not generally planted outside its North American range.

#### A. Deployment of reforestation materials

White Spruce has a long history as a preferred species for reforestation across its range. In the early years, most planting stock were produced as seedlings or transplants in bareroot nurseries (Stiell 1976). Following developments in nursery technology, most planting stock for White Spruce are now produced from seed in containerised systems, in soil-less growing media. A variety of containers are used and stock is raised in both heated and unheated greenhouse structures. Cultural techniques have become highly sophisticated, ensuring that high-quality planting stock can be produced reliably and efficiently (Landis *et al.* 1989, 1990a, b, 1992, 1995).

White Spruce planting stock can also be produced by means of vegetative propagation. The simplest approach is to bulk-up (vegetatively multiply) tested crosses between selected individuals. White Spruce cuttings taken from the seedlings can then be rooted to produce stecklings for deployment (Russell and Ferguson 1990).

Techniques for the initiation and regeneration of somatic embryos have been available for White Spruce for about 10 years (*e.g.*, Hakman and Fowke 1987; Lu and Thorpe 1987; Hakman and von Arnold 1988). In fact, work on *Picea glauca* has been responsible for many *in vitro* technologies now used with coniferous plants. Since then, technical progress has been rapid, and the production of White Spruce planting stock is also now possible by means of somatic embryogenesis. In addition to *P. glauca* somatic embryogenesis has also been achieved in *P. engelmannii* and in *P. glauca engelmannii* complex (Wilson and Thorpe, 1995). Work by Lulsdorf *et al.* (1993) also describes the development of encapsulation of somatic embryos. While embliing production systems have not yet achieved operational status in White Spruce, nursery and field testing has demonstrated that performance of embliings is comparable to that of seedling stock (Grossnickle and Major 1994a, b; Grossnickle *et al.* 1994). Embryogenic lines can be successfully regenerated after cryostorage (Cyr *et al.* 1994; Park *et al.* 1994), making it possible to maintain genotypes in a completely juvenile condition during clonal

testing. White Spruce embaling propagation systems are also being automated further through the application of bioreactor technology to produce "synthetic" seeds (Attree *et al.* 1994).

The advancement of *in vitro* propagation of *P. glauca* has played an important role in the more recent success in genetic transformation of the species. *P. glauca* was the second coniferous species to be stably transformed (Ellis *et al.* 1993). The number of stably transformed coniferous species remains rather low (five to ten species).

The use of direct seeding as a regeneration technique for White Spruce has fluctuated, and results have been erratic (Waldron 1974; Stiell 1976). Its operational use has been largely restricted to Alberta, where direct seeding of White Spruce is often performed as a species mixture with *Pinus contorta* (Kuhnke 1989).

### **B. Provenance transfer**

A long history of provenance tests has demonstrated the general wisdom of using local White Spruce seed sources in the absence of tested alternatives. While some movement of genetic material from point of collection to site of establishment is inevitable, these transfers are normally controlled either a set of transfer rules, regulating distance of movement, or seed zones, where it is assumed that adaptation of populations has been shaped by climate and other ecological factors (Morgenstern 1996). Both are appropriate for a species like White Spruce, where genetic variation is predominantly clinal. Seed zones have been most commonly used throughout the range of White Spruce, where they are normally based on ecological classification schemes (*e.g.*, Fowler and MacGillivray 1967; Konishi 1979). With provenance test data in hand, White Spruce seed zones may be revised to recognise the amplitude of genetic variation and stability over regions (Govindaraju 1990).

While local seed sources are generally recommended for White Spruce, some provenance transfers have been demonstrated to be particularly promising. Provenances from the Ottawa Valley region have continued to perform better than local sources in several field experiments, to the point that breeding programmes based on this material have been recommended in the Lake States (Nienstaedt and Kang 1983), New Brunswick (Fowler 1986) and Quebec (Beaulieu 1996).

### **C. Breeding programmes**

With experimental evidence that substantial genetic variation was to be found within populations, selection of plus-trees has been a common starting point for most improvement programmes. The actual improvement realised through plus-tree selection systems may vary considerably, depending on the techniques used and the stand situations, all of which affect selection intensity, genetic variance and heritability for traits of interest (Morgenstern and Mullin 1988; Cornelius 1994). In White Spruce it is particularly important that sampling of the founder population by selection be balanced, and that breeding strategies maintain this balance to avoid rapid loss of genetic diversity in the breeding population (Nienstaedt and Kang 1987).

Breeding programmes are now well established throughout the range where White Spruce is planted. Regional breeding strategies have been prepared which generally utilise a system of progeny testing and recurrent selection for generation advancement, combined with clonal seed orchards for production of improved seed and usually involving multi-agency co-operation (*e.g.*, Carter and Simpson 1985; Fowler 1986; Dojack 1991; Lamontagne 1992; Stine *et al.* 1995). Flowering of young White Spruce grafts can be stimulated by means of various cultural treatments, particularly those involving gibberellin A<sub>47</sub>, and this has facilitated the turnover of breeding cycles (Greenwood *et al.* 1991; Daoust *et al.* 1995).

Most seed orchards currently in production were established by grafting cuttings from plus-trees, and establishment in cultivated field environments. Early data indicated that such orchards would average over 1 million viable seeds per hectare by the time they entered their productive period (Nienstaedt and Jeffers 1970; McPherson *et al.* 1982). Many of these first-generation orchards are now in production, and some regional nursery requirements are now met completely by orchard seed. Some programmes have also experimented with the management of containerised White Spruce orchards. While container orchards can be conveniently managed to maximise genetic value and to promote flower production, the yield has seldom been more than 10-15 filled seed per cone and requires further development of cultural protocols (Webber and Stoeck 1995).

#### D. Conservation of genetic resources

Domestication of a key species such as White Spruce can influence diversity of genetic resources (1) indirectly, through the method of seed collection, extraction and storage, and through nursery and plantation culture; and (2) directly, through intentional selection to increase the frequency of genes for desirable traits (Morgenstern 1996). The inadvertent loss of genes through natural processes and human activity can have negative consequences for the adaptability of populations and the potential for future gains from breeding.

Throughout most of the range of White Spruce, *in situ* conservation of genetic resources is practised by protecting of ecological reserves, special areas and parks (Pollard 1995), and is integrated with domestication activities that control the movement of seed, active management of existing stands to maintain biological diversity, and protection of small isolated populations (VanBorrendam 1984; Dhir and Barnhardt 1995; Villeneuve 1995; Yanchuk 1995). As outcrossing rates in White Spruce stands can be lower than those of other conifers, inbreeding depression related to population size is a concern for *in situ* conservation efforts. Studies have been initiated to develop guidelines on minimum viable population size (Mosseler *et al.* 1995).

*Ex situ* conservation, through cryopreservation of germplasm, off-site maintenance of populations in arboreta and clone banks, and multi-population breeding strategies (Eriksson *et al.* 1993; Namkoong 1995), has been practised to a much lesser extent, although many White Spruce provenances and families are now represented in field tests and seed bank collections (Plourde *et al.* 1995). Such "active" forms of gene management must be accelerated in preparation for response to rapid environmental and climate changes (Ledig and Kitzmiller 1992).

#### 2. Taxonomy

White Spruce (*épinette blanche* in French Canada) is one of about 40 species of the genus *Picea* A. Dietr. (family Pinaceae) distributed throughout the cooler parts of the North Temperate Zone and higher elevations in the south, and one of seven species native to North America and five native to Canada (Farrar 1995). Its scientific name is now well recognised as *Picea glauca* (Moench) Voss, although it has also been referred to in the literature under an array of botanical synonyms including *Picea canadensis* B.S.P. and *Picea alba* Link. (Sutton, 1970; Krüssmann, 1985). Its colloquial synonyms are even more numerous. They include cat spruce, skunk spruce and Canadian spruce in English, and *épinette à bière*, *épinette des champs* and *sapinette blanche* in French (Sutton 1970).

A variety is generally recognised as Porsild spruce (*Picea glauca* var. *porsildii* Raup) in northern Alberta, the Yukon and Alaska (Farrar 1995). Introgressive hybridisation between white and Englemann spruce (*Picea engelmannii* Parry ex Engelm.) is common where the two are sympatric in western Canada, Montana and Wyoming, and the hybrids have given rise to a variety known as *Picea glauca* var. *albertiana* (S. Brown) Sarg. (Roche 1969; Roche *et al.* 1969; Daubenmire 1974).

Introgressive hybridisation between white and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) also occurs in sympatric areas in northwestern British Columbia and Alaska, with the hybrid known as *Picea* × *lutzii* Little (Roche 1969; Copes and Beckwith 1977; Yeh and Arnott 1986). A rare natural hybrid between white and black spruce (*Picea mariana* (Mill.) B.S.P.), known as “Rosendahl” spruce, has been recognised in the southern part of the range (Little and Pauley 1958; Riemenschneider and Mohn 1975) and has been reported as occurring commonly in northwestern Canada (Larsen 1965; Roche 1969), but its F1 hybrid status has been questioned (Parker and McLachlan 1978). Many named horticultural varieties are recognised (Krüssmann 1985; Griffiths 1994).

There is lack of agreement among taxonomists regarding the subdivision of the genus *Picea* (Schmidt-Vogt 1977). Most early taxonomists suggested dividing the genus into three sections: Eupicea (or Morinda), Casicta and Omorika. Mikkola (1969) recommended recognition of only two sections: Abies and Omorika. After extensive crossability studies, Fowler (1983, 1987a) has suggested that the section Omorika be further divided into two subsections, Omorikoides and Glaucoides, with White Spruce assigned to the latter together with Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Engelmann spruce (*Picea engelmanni* Parry).

### 3. Centres of Origin/ Diversity

#### A. Natural distribution

The natural range of White Spruce extends from the Atlantic to within 100 km of the Pacific Ocean, and from the northern tree limit across North America south into northern New England, New York and the Lake States (Sutton 1970). Over this tremendous range it is found at elevations ranging from sea level to 1 520 m (Nienstaedt and Zasada 1990). Alone or with black spruce and tamarack (*Larix laricina* (Du Roi) K. Koch), White Spruce forms the northern limit of tree-form growth. Outlier populations have been reported as far south as the Black Hills in Wyoming and South Dakota (Sutton 1970).

Several range maps have been prepared for White Spruce, but that drawn by E.L. Little, Jr. and presented in Fowells (1965) has formed the basis for maps found in current reference publications (Nienstaedt and Zasada 1990; Farrar 1995). Little's map is shown in Figure 3.6.

#### B. Evolution and migrational history

Fossil records indicate that divergence of genera in Pinaceae occurred some 135 million years ago during the late Jurassic or early Cretaceous period (Florin 1963). Based on comparative immunological studies, Prager *et al.* (1976) have suggested that *Picea* was among the first genera to emerge.

Although not supported by fossil evidence, Wright (1955) suggested eastern Asia as the likely origin of *Picea*, based on the abundance of species and particularly the presence of *Picea koyamai* Shirasawa, which he felt is a primitive species. *Picea* is then thought to have migrated to North America in one or more waves via a land bridge between Siberia and Alaska (Wright 1955). Critchfield (1984) cites fossil evidence that the White Spruce extended in a broad, shifting pattern across much of North America by the Late Pleistocene.

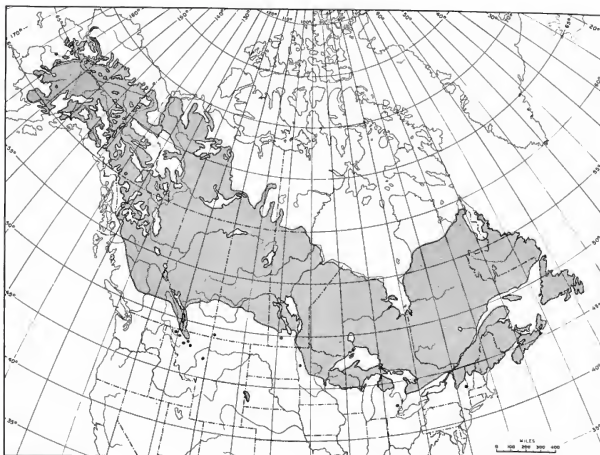
Phylogenetic relationships within coniferous genera are commonly interpreted from species crossability studies, where it is assumed that the more related are two species, the more easily they can be crossed (Wright 1955; Critchfield 1973). The close phylogenetic relationship between the northwestern American “white” spruces (white, Sitka and Engelmann spruce) and the eastern Asiatic

*P. jezoensis* (Sieb et Zucc.) Carr. (Wright 1955; Roche and Fowler 1975) supports this theory, at least for the members of subsection *Glaucoides* in section *Omorika*.

Fossilised cones of an extinct species, *Picea banksii*, found on Banks Island in Arctic Canada can only be distinguished from those of White Spruce on the basis of mean size. These provide evidence that White Spruce, or a close ancestor such as *P. banksii*, was the link between North America and Asia, rather than *P. jezoensis* (Hills and Ogilvie 1970).

Radiocarbon evidence suggests that White Spruce was likely found at least 280 km further north during the Climatic and Little Climatic Optima, 3 500 and 900 years ago (Sutton 1970). During the Pleistocene glaciation, a main eastern refugium extended further south into the Great Plains and perhaps as far as Lee County, Texas (Patzger and Tharp 1943; Graham and Heimsch 1960), and into North Carolina (Frey 1951). Meanwhile, western refugia are considered to have existed in the Yukon-Alaska and the lower eastern slopes of the Rockies, joined by a “fluctuating corridor” through Alberta (Nienstaedt and Teich 1972). It is considered that these east and west populations then followed the retreat of the ice sheet, meeting in the Great Lakes region (Halliday and Brown 1943; Löve 1959).

**Figure 3.6 The natural range of White Spruce**



Source : Fowells, 1965

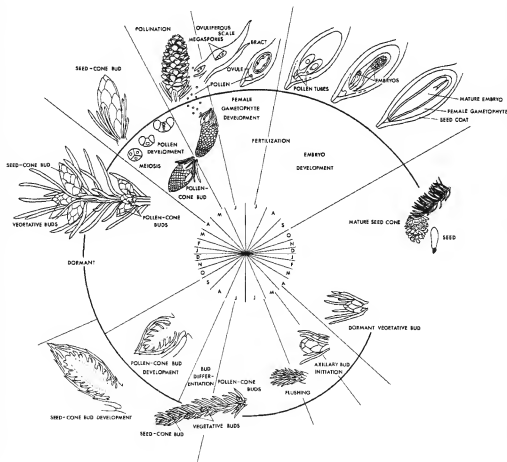
## 4. Reproductive Biology

### A. Reproductive development

White Spruce is monoecious. Development of the reproductive structures follows a two-year cycle typical of most conifers in the northern hemisphere (Figure 3.7), other than *Pinus* species and members of the Cupressaceae family (Owens and Blake 1985). Bud scales are initiated at the terminal apex, and at newly initiated axillary apices within the enlarging vegetative buds, from about late April (Owens *et al.* 1977). Apices differentiate as vegetative, pollen cone or seed cone buds around mid-July, at the cessation of shoot elongation. The proportion of apices differentiating as reproductive buds may be increased by hot, dry weather at the time of differentiation, particularly if preceding cone crops have been poor (Owens and Blake 1985; Nienstaedt and Zasada 1990).

Pollen cone bud development is complete by early October, when they become dormant. Completion of seed cone and vegetative bud development follows shortly thereafter. By the time buds become dormant, all microsporophylls, microsporangia, bracts and functional ovuliferous scales, and leaves have been initiated. Megaspore mother cells are present in the dormant seed cone buds, although meiosis has not begun (Owens and Molder 1977, 1984). Overwintering vegetative buds are small and dome-shaped. Reproductive buds, usually terminal or subterminal, may be identified by their larger size and ovate to obovate shape. Distinguishing between male and female buds may be difficult without dissection, but males are generally found in the middle to lower crown (Eis 1967a; Eis and Inkster 1972).

**Figure 3.7 The reproductive cycle of White Spruce**



Source : Owens and Molder, 1984

Reproductive and vegetative buds break dormancy at about the same time, in response to photoperiod, while subsequent development is regulated by temperature. Meiosis and subsequent development of pollen occur immediately, followed by maturation of the megagametophyte. The developmental morphology of reproductive structures was well-documented with colour photographs by Ho (1991). Flushing of reproductive buds precedes that of vegetative buds, and pollen is released over a one-week period. The pollen enters receptive seed cones and adheres to the sticky micropylar arms. When the cones close, a "pollination drop" draws the pollen into the ovule (Owens and Molder 1979; Ho 1984; Runions *et al.* 1995). Transport of the pollen to the micropyle may be facilitated by rainwater (Runions and Owens 1996). Fertilisation occurs three to four weeks later, and embryo development is completed in early to late August (Owens and Molder 1979; Zasada 1988). Female gametophytes may abort at many stages of development, but most commonly at or shortly after meiosis (Owens and Molder 1984). Without fertilisation, no embryo is formed and the megagametophyte tissue degenerates, leaving a normal-sized but empty seed (Owens and Molder 1979).

## B. Mating system and gene flow

White Spruce is a wind-pollinated, monoecious species, and outcrossing is by far the most prevalent mating system. Self-pollination occurs to some degree, as the period of pollen release and female receptivity coincide for an individual tree (Nienstaedt 1958). Female strobili are concentrated in the top quarter of the crown, while males are more prevalent in the mid to lower crown, but the effectiveness of this zonation against selfing is questionable (Nienstaedt and Teich 1972). The two-step pollination mechanism, whereby pollen is collected in the sticky micropylar arms over the receptive period, and only then drawn en masse by the pollination drop, ensures that pollen from many sources has a chance to fertilise any given ovule (Runions *et al.* 1995). Under controlled pollination, where large quantities of pollen are applied at one time, the micropylar arms become fully occupied and it is more likely that the first-on become first-in (Franklin 1974; Ho 1985). The nucellus itself can accommodate two or three pollen grains, and there is potential of some postzygotic selection to reduce the number of selfed embryos (Fowler 1987b). The incidence of selfing also varies greatly as a function of population size and structure. Selfing rates in a White Spruce seed orchard varied from 0 to 22% (Denti and Schoen 1988). However, the presence of several ramets of the same clone might generate a pollen cloud quite unlike that in a natural stand. Although outcrossing pollen has no advantage over self-pollen prior to fertilisation and self-fertilisation, can occur, most selfed embryos fail to develop, likely as a result of homozygous lethal recessive genes (Mergen *et al.* 1965; Nienstaedt and Teich 1972).

Natural inbreeding among related trees is common in small populations. In two such stands in New Brunswick, the average inbreeding coefficient was estimated as  $F = 0.145$  (Coles and Fowler 1976; Park *et al.* 1984). An electrophoretic study of six stands near the limit of the range in subarctic Quebec displayed deficiencies of heterozygotes at 60% of the loci analysed and suggested rapid accumulation of inbreeding. Differences among these isolated populations were large ( $F_{st} = 0.113$ ), suggesting that genetic drift might be important (Tremblay and Simon 1989). Substantial deviations from random mating and high rates of inbreeding were also observed within stands in Newfoundland (Innes and Ringius 1990) and in a seed production area in Alberta (King *et al.* 1984). Another electrophoretic study of trees throughout a 19 ha stand in eastern Ontario demonstrated that, while selfing was not a major component of the mating system and there was an excess of heterozygotes in both the parental and filial populations, other forms of non-random mating among relatives and restricted transmission distances of effective pollen gametes were important (Cheliak *et al.* 1985).

Gene flow in *Picea* is mediated by small pollen grains, 70–85 nm at their widest point (Eisenhut 1961), whose bladdery wings make them well-adapted for aerial transport (Di-Giovanni and Kevan 1991). Various studies of pollen dispersal in conifers indicate that over 90% of the pollen comes to rest less than 100 m from the source (Wright 1976). Nevertheless, a substantial quantity may travel great distances; Gregory (1973) cites reports that pollen of *Pinus* and *Picea* may travel as far as 600 to 1 000 km, and several authors have concluded that isolation distances of less than 1 km often have little impact on contamination rates in conifer seed orchards (see review by Di-Giovanni and Kevan 1991). A recent study of pollen dispersal dynamics in a black spruce seed orchard indicated that "large amounts" of pollen rose to a height of 300 m above ground level (Di-Giovanni *et al.* 1996). At a steady wind speed of  $5 \text{ m s}^{-1}$ , the authors calculated that spruce pollen reaching this altitude would drift about 47 km. Another study examined pollen contamination within a White Spruce seed orchard that a forest fire had isolated from native stands by as much as 3 km upwind (Caron *et al.* 1994; Mercier *et al.* 1994). In a heavy pollen year, contamination levels within this well-isolated orchard were estimated at 93%.

### C. Seed production

Cones and seeds may be produced on White Spruce as young as four years (Sutton 1970), but most trees do not produce seed until 10 to 15 years. Significant seed production normally occurs on trees that are at least 30 years of age (Nienstaedt and Teich 1972). Periodicity of seed production and crop size are extremely variable. Good cone crops are borne irregularly, but on average every four years (Stiell 1976). In a "heavy" crop year, Waldron (1965) estimated that a mature stand in Manitoba produced 13.8 million seeds/ha, but that only 59% were sound. In a "moderate" seed year, the stand produced 2.5 million seeds/ha. Maximum seed production over a 13-year period in a stand in Alaska was 40 million seeds/ha, and in three years exceeded 10 million seeds/ha (Nienstaedt and Zasada 1990).

Initiation and duration of seed dispersal are weather and site dependent (Zasada 1988). The mature cones open as they lose moisture and the scales flex in dry weather, reclosing during wet periods. Seed dispersal begins in mid- to late-August, with most seeds released in September (Crossley 1953; Waldron 1965; Dobbs 1976). The interval between seed ripening and beginning of dispersal can be less than two weeks, which creates problems in determining the best time to collect cones (Smith 1983; Mercier and Langlois 1992). At some northern sites, seed dispersal may begin before seeds are fully mature (Mercier 1994). In practice, cone collection can begin a couple of weeks earlier if seeds are allowed to "artificially ripen" by storing the cones under cool, moist conditions (Winston and Haddon 1981; Caron *et al.* 1990, 1993).

The seeds are winged and wind-dispersed. The actual distance reached from the source varies from site to site (Dobbs 1976), but in one study less than 5% of the seeds were dispersed more than 100 m from the source (Zasada and Lovig 1983). The seeds themselves are small, and average cleaned seed weight is about 2.0 g/1 000 seeds (Safford 1974).

### D. Natural regeneration

White Spruce seeds exhibit varying degrees of dormancy that may be broken by exposure to low temperatures under moist conditions, *i.e.*, cold stratification (Wang 1974). Dormancy results from inhibition of embryo development, induced by the seed coat and/or megagametophyte tissue (Downie and Bewley 1996). Seed dormancy may vary greatly among stands, individual trees and crop year (Hellum 1968; Wang 1976; Caron *et al.* 1990). In the wild, White Spruce seeds normally germinate the following spring, as soon as soil surface temperatures are warm enough and provided there is adequate moisture (Nienstaedt and Zasada 1990).

Natural regeneration of White Spruce can be difficult to predict and is not easily established under most harvesting systems. As a shade-tolerant species, White Spruce is able to regenerate under mature stands of spruce and early successional species, but advance regeneration stocking is often poor (Jablanczy 1967, 1979; Krasny *et al.* 1984; Walker *et al.* 1986; Nienstaedt and Zasada 1990). Freshly disturbed areas with exposed mineral soils offer the best conditions for germination and establishment (Eis 1967b; Lees 1970; Dobbs 1976; Zasada *et al.* 1978). Thick organic layers are common under mature stands, but such surfaces restrict germination success and shallow root penetration leads to mortality if the canopy is opened suddenly (Jablanczy 1967). Where advance regeneration is established on thick moss, survival after logging is often poor and seedlings are soon replaced by other more aggressive species. Under the more open canopies of stands growing on alluvium sites, the sudden increase of competing vegetation after harvesting prevents seedling establishment and causes severe mortality to advance regeneration (Eis 1981). Advance regeneration that does establish under closed canopies will not survive suppression as long as in other more tolerant

species such as balsam fir (Jablanczy 1967, 1979). Allelopathic effects of *Cladonia* lichens may inhibit the establishment of regeneration (Fisher 1979).

### E. Vegetative reproduction in nature

While vegetative reproduction is rare over much of the range of White Spruce, layering is common at the northern limit where regeneration from seed is limited because of climatic conditions (Nienstaedt and Zasada 1990; Fayle and Scott 1995). Rooting occurs when lower branches of open-grown trees come in contact with the ground and are covered by soil or organic materials. Populations in these arctic areas likely originated from seed at a time when climatic conditions were warmer, and vegetative propagation is now the only possible means of regeneration (Elliott 1979).

## 5. Genetics

### A. Cytology

Vegetative cells are normally diploid, with  $2n = 24$  chromosomes (Mehra and Khoshoo 1956; Santamour 1960). Aneuploidy and polyploidy are very rare (De Torok and White 1960); about 1 in 13 000 seedlings have been observed, mostly tetraploid, and most of them stunted (Winton 1964).

### B. Genetic variation

#### *Population-level variability*

White Spruce was an early candidate for provenance research, and evidence of clinal variation for height growth related to latitude and elevation of origin appeared as early as 1950 (Morgenstern 1996). North-south adaptive variation has also been observed for such characters as cold hardiness of buds, foliage and stems (Simpson 1994), optimal and threshold germination temperature (Fraser 1971), germination rate (Roche 1969), seed quality (Khalil 1986), juvenile growth (Dunsworth and Dancik 1983; Khalil 1986), date of bud flush (Blum 1988), and various other seedling morphological and phenological traits (Nienstaedt and Teich 1972).

There is evidence of east-west variation patterns in such taxonomic characteristics as needle colour, number of stomata and branch pubescence (Nienstaedt and Teich 1972), cortical monoterpenes (Wilkinson *et al.* 1971), DNA content (Miksche 1968) and cpDNA allele frequencies (Furnier and Stine 1995). These data are consistent with the two-refugia theory of White Spruce remigration, following the Pleistocene glaciation, with a major division at about 95°W, with latitudinal clines within each division (Nienstaedt and Teich 1972).

While White Spruce generally exhibits clinal variation for adaptive traits, edaphic ecotypes have been identified in eastern Ontario that produce superior height growth on granitic and limestone sites (Teich and Holst 1974; Murray and Skeates 1985). White Spruce populations from moist-warm habitats of the sub-boreal spruce biogeoclimatic zone in the interior of British Columbia have displayed greater resistance to white pine weevil (*Pissodes strobi*) attack (Alfaro *et al.* 1996). In a 20-year-old trial in the badly polluted industrial Erzgebirge region of East Germany, there was great variation in performance of 16 tested White Spruce provenances. The best of these (from Sundridge, Ontario) was superior to the best of 17 tested provenances of Norway spruce (Weiss *et al.* 1988).

Local provenances are generally well-adapted and grow well, but it is not uncommon for provenances from more southerly locations to exhibit better growth (Nienstaedt and Teich 1972). Some particular sources have demonstrated superior performance over a wide range of sites. A

provenance from Birch Island, British Columbia, has proven exceptional and in coastal nurseries will match the growth of Sitka spruce (Nienstaedt and Zasada 1990). Provenances from the Ottawa Valley have performed well at many locations from the Lakes States through to Newfoundland (Nienstaedt 1969; Corriveau and Boudoux 1971; Teich *et al.* 1975; Fowler and Coles 1977; Radsliiff *et al.* 1983; Khalil 1985). Although these sources grew well in Newfoundland, survival was sometimes poor (Hall 1986). In Nova Scotia, Ottawa Valley sources were surpassed in height growth by provenances from Prince Edward Island (Bailey 1987). In a range-wide provenance test in Alberta, the 10 best provenances included sources from Saskatchewan, Manitoba, Ontario and Quebec, and had 15% greater height and only slightly lower survival after 15 years than did the local seed sources (Hansen *et al.* 1995).

When provenances of diverse geographic origin are tested, population differences may explain 10 to 15% of the phenotypic variation in wood relative density (Stellrecht *et al.* 1974; Beaulieu and Corriveau 1985; Corriveau *et al.* 1987). However, population differences within a smaller geographic area can be negligible, even while family differences within stands can account for 16% of the variation in relative density of outer wood (Corriveau *et al.* 1991).

In contrast to many other characters, geographic variation at polymorphic allozyme loci appears to be weak. A 19-year-old test in Minnesota of 22 provenances from across the range of White Spruce demonstrated that while 48.0% and 54.1% of the variation in height at ages nine and 19, respectively, was due to differences among populations, an average of only 3.8% of the allozyme variation was due to population differences (Furnier *et al.* 1991). The variance among enzyme systems at 13 loci in four populations in Alaska, on an altitudinal gradient from 120 to 750 m, was such that only 2% of the variance was among populations, while 97% of the genetic diversity was within-stand, suggesting that the allozyme systems studied were selectively neutral (Alden and Loopstra 1987).

#### *Individual-level variability*

While variation among provenances is important in determining the risks and benefits of transferring seed sources, genetic improvement from mass selection relies primarily on variation within populations as the source of genetic gains. The partitioning of genetic variance among and within populations is greatly influenced by the range of adaptive variation sampled by the tested provenances. A wide-range sample of provenances tested in Wisconsin estimated population variance to be two to three times the family-within-population variance for height at nine and 15 years (Nienstaedt and Riemenschneider 1985). Another sample drawn from across Quebec and Ontario indicated that population variance was as large as that of families-within-populations (Li *et al.* 1993). Field trials using hierarchical sampling over a limited area of southeastern Ontario showed high within-stand variation for height growth and phenology, while variation among stands was low (Dhir 1976; Pollard and Ying 1979a, b).

The oldest White Spruce progeny tests were established on four sites at the Petawawa Forest Experiment Station in 1958 (Holst and Teich 1969). Narrow-sense heritabilities for this material were reported at age eight to 11 years in the range of 0.15 to 0.35 for height, and at three of the four sites was similar at age 22 (Ying and Morgenstern 1979). A similar progeny test in Minnesota produced heritability estimates for height of 0.27 at age nine, increasing to 0.35 at age 12 (Mohn *et al.* 1976), and another in Wisconsin produced estimates of 0.16 and 0.25 at ages nine and 15, respectively (Nienstaedt and Riemenschneider 1985).

Heritability estimates for diameter have typically been lower: from 0.05 to 0.10 at age 22 in the Ontario test (Ying and Morgenstern 1979), and 0.14 in the Minnesota test (Merrill and Mohn 1985).

The Petawawa trial was revisited by Magnussen (1993), who found that heritability estimates were much higher when only the "crop" trees were considered, as would be the case during selection in older stands. Stem analysis of almost 300 trees from 18 open-pollinated families at 36 years of age showed strong heritability of height growth, in the range of 0.3 to 0.6. Heritability for volume was also moderately strong, peaking at about 0.3 at age 20 and declining rapidly thereafter.

White Spruce in the western part of the range is frequently deformed by the white pine weevil (*Pissodes strobi*). Resistance to this pest varies substantially among individuals within a population, and the genetic basis has been demonstrated (Kiss and Yanchuk 1991; Alfaro *et al.* 1996).

While significant variation in wood specific gravity exists among populations, there are few correlations with environmental gradients or growth, and most of the variability exists among individuals within stands (Beaulieu and Corriveau 1985). Corriveau *et al.* (1991) studied the variation of wood quality characters in 19-year-old open-pollinated progenies from eight populations of White Spruce in the Upper Ottawa Valley. Their results indicated that the relative density of outer wood in White Spruce is under strong genetic control, with 16% of the variation explained by family differences and narrow-sense heritability estimated to be 0.63. An open-pollinated family test in British Columbia produced a similarly high estimate of heritability for wood specific gravity, 0.47, at age 15 (Yanchuk and Kiss 1993).

Substantial genetic variation has also been demonstrated in the initiation, maturation and germination of somatic White Spruce embryos from zygotic embryonic tissue (Park *et al.* 1993, 1994). Of particular concern to clonal selection programmes, a substantial portion of the genetic variance in the response to cultural treatments and the maturation and germination of somatic embryos was due to non-additive genetic variance.

### C. Inbreeding depression and genetic load

Strong inbreeding depression has been reported in White Spruce (Mergen *et al.* 1965; Fowler and Park 1983; Park *et al.* 1984), and height growth losses as great as 33% have been reported (Ying 1978). Compared with other conifers, the number of lethal equivalents per zygote, 12.6, is high, and selfing has severe effects on seed set, early growth and survival (Fowler and Park 1983). Selection likely acts to remove selfed and highly inbred individuals early in the life-cycle, prior to the age of reproduction (Furnier *et al.* 1991). Nevertheless, natural inbreeding among related trees is common in small populations. In two such stands in New Brunswick, the average inbreeding coefficient was estimated as  $F = 0.145$  (Coles and Fowler 1976; Park *et al.* 1984).

## 6. Crosses

Potential crosses with White Spruce are summarised in Table 3.13 (modified from Nienstaedt and Teich 1972). Introgressive hybridisation between white and Englemann spruce is widespread where the species are sympatric over large areas of British Columbia and Alberta (Nienstaedt and Teich 1972). In these areas breeding programmes simply treat the hybrid complex as a single species, "interior spruce".

Table 3.13 Species cross compatibility with White Spruce

Species	References
<b>Commonly occurring in sympatric range</b>	
<i>P. englemannii</i> Parry ex Engelm. = <i>P. glauca</i> var. <i>albertiana</i> (S. Brown) Sarg.	Roche 1969; Daubenmire 1974
<i>P. sitchensis</i> (Bong.) Carr. = <i>Picea</i> × <i>lutzii</i> Little	Roche 1969; Fowler 1987a
<b>Successful crosses; hybridity verified</b>	
<i>P. jezoensis</i> var <i>hondoensis</i> (Mayr.) Rehder	Wright 1955
<i>P. koyamai</i> Shirasawa	Wright 1955
<i>P. omorika</i> (Pancic) Purkyne	Jeffers 1971; Gordon 1980
<i>P. pungens</i> Engelm.	Hanover and Wilkinson 1969; Bongarten and Hanover 1982; Gordon 1980
<i>P. schrenkiana</i> Fisch. & Mey.	Fowler 1966; Gordon 1980
<b>Limited crossability; hybridity verified</b>	
<i>P. likiangensis</i> (Franch.) Pritz.	Jeffers 1971; Gordon 1986
<i>P. maximowiczii</i> Reg.	Jeffers 1971
<i>P. mexicana</i> Martinez	Gordon 1980
<i>P. mariana</i> (Mill.) B.S.P.	Gordon 1986; Little and Pauley 1958; Parker and McLachlan 1978
<i>P. smithiana</i> Boiss.	Mergen <i>et al.</i> 1965; Nienstaedt and Fowler 1982
<b>Possible crossability; hybrids not verified</b>	
<i>P. abies</i> (L.) Karst.	Jeffers 1971
<i>P. asperata</i> Mast.	Mergen <i>et al.</i> 1965
<i>P. chihuahuana</i> Martinez	Gordon 1980
<i>P. glehnii</i> (Fr. Schmidt) Mast.	Anonymous 1962
<i>P. montigena</i> Mast.	Jeffers 1971
<i>P. orientalis</i> (L.) Link	Mergen <i>et al.</i> 1965
<i>P. retroflexa</i> Mast.	Jeffers 1971
<i>P. rubens</i> Sarg.	Gordon 1980; Bongarten and Hanover 1982

Source : Modified from Nienstaedt and Teich, 1972

## 7. Ecology and Associated Species

Much of the information in this section has been derived from the excellent chapter on the silvics of White Spruce by Hans Nienstaedt and John Zasada, in USDA Forest Service Agricultural Handbook 654 (Nienstaedt and Zasada 1990). Other citations are given as appropriate when specific information is attributable to other sources.

### A. Habitat

Having repopulated a tremendous area following glaciation, White Spruce can grow under a great variety of conditions, including extreme climates and soils, and is regarded as a "plastic" species. It is tolerant of shade, but recovers well after release from suppression and exposure to more light (Farrar 1995). Although it is a climax species in succession, it not only succeeded in establishing itself soon after glaciation, but also demonstrated an ability to invade abandoned farmland throughout eastern Canada, occupying about 200 000 ha of old fields in Nova Scotia alone (Drinkwater 1957).

### Climate

The northern limit of the White Spruce is likely determined by a number of climatic, biotic and abiotic factors. What is clear is that climatic extremes in this area are significant. Mean daily

temperatures for January throughout much of the species range in Alaska, the Yukon and the Northwest Territories are in the vicinity of -29°C, whereas those in July reach only 13°C. Moisture is also limited in this area, with mean annual precipitation of only 250 mm. While photoperiod north of the Arctic Circle is 24 hours at the summer solstice, the length of the growing season at the northern limit is only about 60 days and may be as short as 20 days.

The southern limit of White Spruce's dominance as a species in forest stands roughly follows the 18°C July isotherm, except in the Prairie Provinces where it swings somewhat north. Maximum summer temperatures as high as 43°C have been recorded within the range in Manitoba, and mean annual precipitation can be as high as 1 270 mm in Nova Scotia and Newfoundland. Low mean annual precipitation in the range of 380 to 510 mm combines with mean high temperatures in July of over 24°C to produce the most severe conditions along the southern edge of the range in the Prairie Provinces.

### *Soils and site type*

A wide range of soils and site conditions support White Spruce, although the diversity of sites becomes more limited in northern areas with increasing severity of climate (Sutton 1970). Within its range, it is found on soils of glacial, lacustrine, marine and alluvial origin derived from geologically diverse substrata, including granites, gneisses, sedimentaries, slates, schists, shales and conglomerates.

Podzolic soils are most common, but White Spruce also grows on brunisolic, luvisolic, gleysolic and regisolic soils. It can also be found as a minor species on sand flats and other coarse-textured soils, on shallow mesic organic soils in Saskatchewan, and on organic soils with black spruce in the central Yukon.

While White Spruce can occupy extremely harsh site conditions, it is generally regarded as more demanding than other associated conifers, requiring higher moisture and fertility to achieve best development on moderately well-drained soils. Optimum pH values are probably in the range of 4.7 to 7.0 (Sutton 1970; Stiell 1976), but White Spruce stands are found on strongly acidic soils at pH 4.0, as well as alkaline soils as high as pH 8.0. Ecotypic variation has been observed in White Spruce, with some ecotypes adapted to limestone sites (Teich and Holst 1974; Murray and Skeates 1985). White Spruce stand development itself can have an impact on organic layers and on properties of the mineral soil. Brand *et al.* (1986) found that soil pH decreased by 1.2 units in plantations established on abandoned farmland in Ontario.

### **B. Synecology and associated species**

Distributed over such a wide range, it is no surprise that White Spruce is an important component of several different forest types. In the eastern part of its range, it occurs in pure stands on abandoned fields in New England and the Maritime Provinces (Drinkwater 1957; Sutton 1970) and in moist boreal regions in the north. It more commonly occurs as a major stand component in association with black spruce (*Picea mariana*), red spruce (*Picea rubens*), balsam fir (*Abies balsamea*), white birch (*Betula papyrifera*) and trembling aspen (*Populus tremuloides*), and to a lesser extent with yellow birch (*Betula alleghaniensis*) and sugar maple (*Acer saccharum*). When White Spruce occurs in communities with intolerant species such as trembling aspen, white birch or red pine (*Pinus resinosa*), its greater shade tolerance leads to its assuming increasing importance as succession progresses. In northern Quebec, White Spruce is associated with lichen (*Cladonia*), feathermosses (e.g., *Hylocomium splendens*, *Pleurozium schreberi*, *Ptilium cristacastrensis*, and *Dicranum* spp.), dwarf birch (*Betula nana*) and many ericaceous plants.

Pure stands are more common in the western part of the range. Associated species in such stands in Alaska include white birch, trembling aspen, black spruce and balsam poplar (*Populus balsamifera*), whereas in western Canada the pure White Spruce type is associated with subalpine fir (*Abies lasiocarpa*), balsam fir, Douglas fir (*Pseudotsuga menziesii*), jack pine (*Pinus banksiana*) and lodgepole pine (*P. contorta*). In northwestern Canada and Alaska, closed White Spruce stands occur in communities with willows (*Salix* spp.) and buffalo berry (*Shepherdia* spp.), combined either with northern goldenrod (*Solidago multiradiata*) and crowberry (*Empetrum* spp.), or with huckleberry (*Gaylussacia* spp.), dewberry (*Rubus* spp.) and peavine (*Lathyrus* spp.).

In low elevations of western Canada and throughout interior Alaska, White Spruce is found in mixed-wood stands with trembling aspen. Common understorey shrubs found under such canopies in Alaska include green alder (*Alnus crispa*), willows, common bearberry (*Arctostaphylos uva-ursi*), highbush cranberry (*Viburnum edule*) and mountain cranberry (*Vaccinium vitis-idaea*). In the Prairie Provinces, the White Spruce-aspen type is associated with common snowberry (*Symphoricarpos albus*), red osier dogwood (*Cornus stolonifera*), western serviceberry (*Amelanchier alnifolia*) and western chokecherry (*Prunus virginiana* var. *demissa*).

Mixed White Spruce-paper birch stands are also common in western Canada and parts of Alaska. In this stand type, the understory vegetation usually includes willows, green alder, highbush cranberry, prickly rose (*Rosa acicularis*), mountain cranberry, bunchberry (*Cornus canadensis*) and Labrador-tea (*Ledum groenlandicum*).

Both the White Spruce-aspen and White Spruce-white birch stand types are successional stages leading to the pure White Spruce type or, in alpine treeline communities, the black spruce-White Spruce type. The latter occurs as open stands that, depending on moisture availability, may also support resin birch (*Betula glandulosa*), alders, willows, feathermosses and *Cladonia* lichens, together with Labrador-tea, bog blueberry (*Vaccinium uliginosum*), mountain cranberry and black crowberry (*Empetrum nigrum*).

Where White Spruce occurs as an important component of the boreal spruce-fir forest, green alder is the most commonly associated tall shrub, with willows important in western areas, and mountain maple (*Acer spicatum*), showy mountain ash (*Sorbus decora*) and American mountain ash (*S. americana*) important in the east. Common medium to low shrubs are highbush cranberry, red currant (*Ribes triste*), prickly rose and raspberry (*Rubus idaeus*). Ground vegetation commonly includes fireweed (*Epilobium angustifolium*), one-sided wintergreen (*Pyrola secunda*), one-flowered wintergreen (*Moneses uniflora*), northern twinflower (*Linnaea borealis*), naked bishop's cap (*Mitella nuda*), bunchberry, dwarf rattlesnake plantain (*Goodyera repens*), stiff club moss (*Lycopodium annotinum*) and horsetail (*Equisetum* spp.) (la Roi 1967). Many bryophytes occur in these boreal spruce-fir stands. The most common mosses are *Pleurozium schreberi*, *Hylacomium splendens*, *Ptilium cristacastrensis*, *Dicranum fuscescens* and *Drepanocladus uncinatus*. Common liverworts are *Ptilidium pulcherrimum*, *P. ciliare*, *Lophozia* spp. and *Blepharostoma trichophyllum*. Common lichens include *Peltigera aphosa*, *P. canina*, *Cladonia rangiferina*, *C. sylvatica*, *C. alpestris*, *C. gracilis* and *Cetraria islandica* (la Roi and Stringer 1976).

### C. Competition and stand structure

White Spruce can exist in various stand types and various stages of succession. Under shade, it is classified as intermediate to tolerant (Nienstaedt and Zasada 1990; Farrar 1995). It will compete with, but not necessarily outperform, other shade-tolerant conifers such as hemlock, black and red spruce, balsam fir, sugar maple and beech. In association with less tolerant early-successional species such as aspen, white birch and lodgepole pine, it may remain a suppressed, understorey component, becoming

more prominent at later successional stages. White Spruce competes poorly against the dense growth of perennials, bracken fern and understorey shrubs (Fowells 1965).

While White Spruce can form pure stands, particularly in the northwestern part of its range and in the New England States and Maritime Provinces, these stands are not always self-sustaining climax types. Where White Spruce pioneers to form even-aged stands on old fields in the Maritimes, advance regeneration is often outnumbered and outperformed by balsam fir seedlings that become a larger component after release (Jablanczy 1979). In mixtures, particularly with less tolerant species, the response to release by disturbance or cutting can be much more successful (Crossley 1976; Berry 1982; Nienstaedt and Zasada 1990).

White Spruce can be a component of multi-aged stands, either as pure stands or mixed with other tolerant late-successional conifers and hardwoods. Older age classes in such stands can be as high as 200 to 250 years in Alberta (Day 1972; Nienstaedt and Zasada 1990). As establishment is facilitated by disturbance, the age distribution in such stands is not continuous, but rather grouped according to periods of successful establishment.

#### D. Ecosystem dynamics

Many abiotic factors interact with White Spruce in forest ecosystems; some pose a direct threat to the species or cause significant damage. Wild fires can eliminate seed supply and leave a seedbed that is more conducive to the establishment of other species such as lodgepole pine, intolerant hardwoods and even black spruce. Stands established on flood plains may benefit from deposit of seedbed materials or suffer from disturbance to young regeneration. Frost heaving can cause severe damage, particularly to container seedlings planted on finer-textured soils. Root form and depth of White Spruce can vary greatly depending on site conditions (Strong and la Roi 1983), and shallow-rooted stands may be prone to windthrow. Periodic storms may cause considerable damage from hail, ice and snow (Dobbs and McMinn 1973; Gill 1974; Sampson and Wurtz 1994). Late-spring frosts can cause significant damage to flushing vegetative and reproductive buds.

While a great number of insects are a natural component of White Spruce forest types, few are responsible for large losses. Of these, the eastern spruce budworm (*Choristoneura fumiferana*) is the most destructive. Massive epidemics of this defoliator occur periodically, resulting in heavy mortality and loss of growth, particularly where White Spruce is associated with balsam fir (Rose *et al.* 1994). Several other defoliators cause damage or weaken trees on a smaller scale, including the yellow-headed spruce sawfly (*Pikonema alaskensis*), European spruce sawfly (*Diprion hercyniae*), needleminers, needleworms, loopers, tussock moths and the spruce harlequin. Other groups of insects attack buds and shoots of White Spruce, including gall-forming adelgids (*Adelges* spp.), spruce bud moths (*Zeiraphera* spp.) and the white pine weevil (*Pissodes strobi*).

Several species of bark beetles, *Scolytidae*, feed and breed in galleries between the bark and wood. The spruce beetle (*Dendroctonus rufipennis*) may attack trees of normal vigour, particularly those which are large-diameter and slow-growing, and has killed large areas (Ostaff and Newell 1981; Hard *et al.* 1983; Werner and Holsten 1984). Generally, though, bark beetles are considered secondary pests, attacking trees weakened by other means such as budworm epidemics, and may be thought of as beneficial in that their feeding hastens the return of wood to the humus (Rose *et al.* 1994).

Warren's collar weevil (*Hylobius warreni*) causes significant damage in scattered areas, girdling smaller trees and making larger trees susceptible to root rots such as *Inonotus tomentosus* (Merler and van der Kamp 1984; Rose *et al.* 1994). The strawberry root weevil (*Otiorynchus ovatus*) can cause injury to young seedlings, and the root-collar weevil *Hylobius congener* can cause significant

mortality to White Spruce seedlings planted on recently cut softwood sites (Pendrel 1990; Eidt and Weaver 1993).

Many insect species inhabit or feed on spruce cones and seed, as part of their life-cycle. Significant losses in natural stands and seed orchards are caused in particular by the White Spruce cone maggot (*Strobilomyia neanthracina*) and the spruce seed moth (*Cydia strobilella*), and to a lesser extent by the spruce budworm and spruce coneworm (*Dioryctria reniculelloides*), among others (Hedlin *et al.* 1980; Turgeon 1994). Only a few pathogens cause problems with cone and seed production. The spruce cone rust (*Chrysomyxa pirolata*) can cause abnormal development of the cones, reduced seed production, and decreased viability of seeds (Sutherland *et al.* 1987; Myren *et al.* 1994).

Emerging seedlings, particularly in bareroot nurseries, are commonly affected by damping-off fungi, primarily *Fusarium* but also *Pythium*, *Rhizoctonia*, *Phytophthora* and *Cylindrocladium* (Filer and Peterson 1975). Young seedlings may also suffer from Sirococcus blight (*Sirococcus strobilinus*) and infestation by the nematode *Xiphinema bakeri* (Sutherland and Van Eerden 1980).

Spruce needle rust (*Chrysomyxa ledi* and *C. ledicola*) is common wherever the alternate host, Labrador-tea, is found, but extensive damage from the fungus is rare. Spruce broom rust is common, causing abnormal proliferation of shoots to form "witches'-broom", but rarely causes death. Witches'-broom on White Spruce is sometimes caused by eastern dwarf mistletoe (*Arceuthobium pusillum*), although black spruce is more susceptible to this parasite. Scleroderris canker (*Gremmeniella abietina*) and cytospora canker (*Leucostoma kunzei*) both affect White Spruce, but cause little damage (Myren *et al.* 1994). Massive tumour-like growths are commonly observed on stems and branches in some White Spruce populations, particularly near coastal areas, but their etiology is not known (De Torok and White 1960).

Many rot fungi produce stem, butt and root rot in White Spruce, including red ring rot (*Phellinus pini*), red belt fungus (*Fomitopsis pinicola*) and Armillaria root rot (*Armillaria mellea* complex). Tomentosus root rot (*Inonotus tomentosus*) and brown cubical rot (*Phaeolus schweinitzii*) infect root systems and can reduce quality and growth, even if direct mortality is often light (Myren *et al.* 1994).

White Spruce forest stands commonly provide cover for many species of animals. Some, like moose, deer, black bear and many other fur-bearers, seek shelter in forest habitats but rarely feed on White Spruce. Porcupines (*Erethizon dorsatum*) also seek shelter in White Spruce forests and may kill small numbers of trees by feeding on the bark (Rose *et al.* 1994). Snowshoe hares (*Lepus americanus*), which commonly feed on foliage of young trees, tend to favour many other conifer species over White Spruce (Bergeron and Tardif 1988; Rangen *et al.* 1994), although planted White Spruce seedlings are preferred over natural regeneration (Sampson and Wurtz 1994). Many small mammals such as squirrels, mice, voles, chipmunks and shrews are heavy consumers of White Spruce seed and can have a major impact on regeneration, while the impact of seed-eating birds, including chickadees, grossbeaks, crossbills, juncos and sparrows, is relatively small (Radvanyi 1974). Many more bird species feed on the many species of insects that inhabit or feed on White Spruce trees and associated species.

The hybrid between white and Sitka spruce, *Picea* × *lutzi*, also occurs naturally where these species are sympatric. The hybrid has frequently been made artificially with parents from outside the sympatric area (Fowler 1987a), often in the hope of imparting the resistance of White Spruce to the white pine weevil (*Pissodes strobi* Peck). The degree of cold hardiness of the hybrid is related to the proportion of White Spruce germplasm (Ying and Morgenstern 1982), and growth performance of the hybrid depends greatly on the origin of the parents (Sheppard and Cannell 1985).

Many other artificial hybrids have been made successfully (e.g. Wright 1955; Jeffers 1971; Bongarten and Hanover 1982). The hybrid with Himalayan spruce (*P. smithiana*) was inferior to native White Spruce when field tested in New Brunswick, but superior in Wisconsin (Nienstaedt and Fowler 1982). Generally speaking, few of these hybrids have shown promise and none has achieved commercial importance (Nienstaedt and Zasada 1990).

## 8. Summary

White Spruce is an enormously important tree species in North America. It occupies a dominant role in several forest types that span the breadth of the continent, from the northern tree limit south to the Lake States and New England. The species has been successful as both a pioneering and climax type, and is genetically broadly adapted and highly variable. It is an outcrossing, wind-pollinated species that can transfer genes rapidly, and yet it tolerates higher levels of inbreeding when found in small populations.

The ecology of White Spruce is extremely diverse, given its tremendous geographic distribution and its genetic plasticity. The typical White Spruce ecosystem has a diverse mixture of associated tree species, vascular flora, bryophytes, insects, fungi, birds and animals. Only a very small number of these associated species pose a major threat by competition or direct damage, and White Spruce is well-adapted to this complex coexistence.

White Spruce is well-suited to artificial regeneration. It is the most commonly planted forest species throughout its natural range. Tree breeding programmes have a long history, and improved material from seed orchards now constitutes a significant portion of deployed reforestation material in some areas. While White Spruce reforestation is currently based on seed propagation, vegetative propagation techniques for cuttings and regeneration of somatic embryos are well-advanced, making it a logical target for implementation of transgenic biotechnologies and the use of cloning in both breeding and deployment strategies.

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*Part 4*

**CONSENSUS DOCUMENTS ON MICRO-ORGANISMS**

## SECTION I BACULOVIRUSES

### I. General Considerations

#### 1. Subject of Document: Species Included and Taxonomic Considerations

##### A. Taxonomic considerations

Baculoviruses are a family of arthropod-specific, rod-shaped (baculum = rod), enveloped viruses with a circular double-stranded DNA genome. Until recently, the family Baculoviridae was divided into two subfamilies, the Eubaculovirinae and the Nudibaculovirinae (Francki *et al.*, 1991). Based on the type of virion occlusion (see below) the Eubaculovirinae comprised the genera (i) nuclear polyhedrosis virus (NPV) and (ii) granulovirus (GV). The subfamily Nudibaculovirinae contained the only genus non-occluded baculovirus (NOB) which differed from the Eubaculovirinae in the lack of occlusion body formation and virion morphology (for review see Burand, 1991).

Recently, the International Committee on Taxonomy of Viruses (ICTV) revised the classification of baculoviruses (Murphy *et al.*, 1995). The family Baculoviridae is now divided into two genera (i) Nucleopolyhedrovirus (formerly nuclear polyhedrosis virus) and (ii) Granulovirus (formerly granulosis virus). The NOB including the *Oryctes rhinoceros* virus (OrV) and the *Heliothis* (= *Helicoverpa*) *zea* virus 1 (HzV-1) has been removed from this family and are not assigned to any virus family.

Some properties used for the taxonomy and classification criteria for baculoviruses are summarised as follows (Murphy *et al.*, 1995) (Fig. 1 in A 2.1).

Baculoviruses exclusively have been isolated from arthropods, primarily from 4 insect orders as Lepidoptera, Hymenoptera, Diptera and Coleoptera (Martignoni and Iwai, 1986b; Adams and Bonami, 1991).

During the replicative cycle of baculovirus, two virion phenotypes are produced. One virion phenotype, called occlusion derived virus (ODV), is embedded into a crystalline protein matrix, the occlusion body. Occlusion bodies are polyhedral and contain numerous virions (genus *Nucleopolyhedrovirus*) or ovoid cylindrical and contain only one (rarely two) virions (genus *Granulovirus*). The ODVs of granuloviruses contain only one nucleocapsid within the viral envelope, whereas NPV ODVs can harbour a single nucleocapsid (SNPV) or multiple nucleocapsids (MNPV) per virion. A second virus phenotype, called budded virus (BV), is generated during early stages of infection. BV consist of single nucleocapsids which bud through the plasma membrane of infected cells into the extracellular fluid. Their membrane envelopes are loose-fitting and contain peplomers of a viral encoded glycoprotein (Fig. 1 in A 2.1).

The rod-shaped nucleocapsids are 30-55 nm in diameter and 250-300 nm in length and contain a single supercoiled, closed circular doublestranded DNA of 90-160 kb.

## B. Species included

Among the 633 potential baculovirus species compiled by the ICTV, 15 NPV were categorised as assigned species whereas 483 NPV are tentative species. The GV contains 5 assigned and 131 tentative species (Table 4.1). In general, the name of a given baculovirus consists of two parts, the name of the host insect where the baculovirus was isolated from and the type of occlusion body formed, e.g. the multiple nucleocapsid nucleopolyhedrovirus of the alfalfa looper *Autographa californica* is termed *Autographa californica* MNPV or AcMNPV.

Table 4.1 List of assigned baculovirus species

Family: Baculoviridae	
1. Genus <i>Nucleopolyhedroviruses</i>	NPV
<i>Autographa californica</i> MNPV (type species)	AcMNPV
<i>Anticarsia gemmatilis</i> MNPV	AgMNPV
<i>Bombyx mori</i> NPV	BmNPV
<i>Choristoneura fumiferana</i> MNPV	CfMNPV
<i>Galleria mellonella</i> MNPV	GmMNPV
<i>Helicoverpa zea</i> SNPV	HgSNPV
<i>Lymantria dispar</i> MNPV	LdMNPV
<i>Mamestra brassicae</i> MNPV	MbMNPV
<i>Orgyia pseudotsugata</i> MNPV	OpMNPV
<i>Orgyia pseudotsugata</i> SNPV	OpSNPV
<i>Rachiplusia ou</i> MNPV	RoMNPV
<i>Spodoptera exigua</i> MNPV	SeMNPV
<i>Spodoptera frugiperda</i> MNPV	SfMNPV
<i>Trichoplusia ni</i> MNPV	TnMNPV
<i>Trichoplusia ni</i> SNPV	TnSNPV
2. Genus <i>Granulovirus</i>	GV
<i>Plodia interpunctella</i> GV (type species)	PiGV
<i>Artogeia rapae</i> GV	ArGV
<i>Cydia pomonella</i> GV	CpGV
<i>Pieris brassicae</i> GV	PbGV
<i>Trichoplusia ni</i> GV	TnGV

Source : Murphy et al., 1995

The subject of this document includes the nucleopolyhedroviruses and granuloviruses with emphasis on those that have been used for insect control. Investigations on potential improvements of application strategies and biological properties predominantly concentrate on species/strains that are infective for lepidopteran hosts.

## 2. Characteristics of the Organism Which Permit Identification, and the Methods Used to Identify the Organism

Baculoviruses form a distinct and well characterised group of arthropod-specific viruses which can be distinguished from other viruses by a number of unique properties described in the following.

### A. Morphological and physicochemical characteristics

The most prominent characteristic of baculoviruses is the formation of occlusion bodies (OB). The OB are formed in the nuclei of infected cells and can be easily detected by light microscopy (phase-contrast or dark-field) as highly refractile particles.

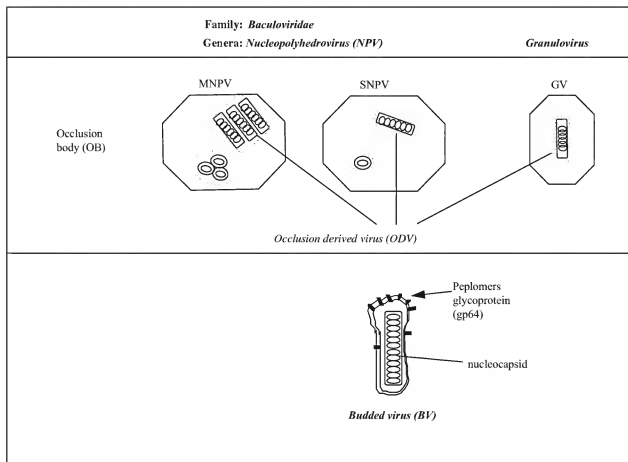
Nucleopolyhedroviruses form polyhedra-like occlusion bodies of 0.15 to 15  $\mu\text{m}$  in size and contain many enveloped virions. The major component of the occlusion body is a single, viral encoded protein of Mr 25-33  $\times 10^3$ , called polyhedrin (Hooft van Iddekinge *et al.*, 1983). Polyhedral occlusion bodies normally band at 54-56% sucrose on 40-65% w/w sucrose gradients at 100,000 g. The buoyant density of ODVs in CsCl is 1.18-1.25  $\text{g}/\text{cm}^3$ , that of BV in sucrose is 1.17-1.18  $\text{g}/\text{cm}^3$ .

Electron microscopic observation of polyhedral inclusion bodies reveal two morphotypes: (i) single nucleocapsid nucleopolyhedroviruses (SNPV) contain only a single nucleocapsid within a virion, whereas the virions of (ii) multiple nucleocapsid nucleopolyhedroviruses (MNPV) harbour few to many nucleocapsids. Factors determining and regulating the formation of SNPV or MNPV have not been elucidated.

Granuloviruses generally form ovicylindrical (granule-like) occlusion bodies of 120-300 nm in width and 300-500 nm in length (Crook, 1991). The matrix protein, called granulins, is genetically and serologically closely related to the NPV polyhedrin.

SDS-polyacrylamide gel electrophoresis and serological techniques such as immunodiffusion, immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and Western-blotting have been used to identify particular NPV and GV and to study their relationship (Summers and Smith, 1975; Reinganum, 1984). ELISA was demonstrated to be a rapid, specific and sensitive method for detecting and quantifying baculoviruses (reviewed by Harrap and Payne, 1979). Polyclonal and monoclonal antisera specific against occlusion body and capsid proteins revealed a high degree of cross-reactivity among NPV and GV (Smith and Summers, 1981). These traits allowed the identification of a single baculovirus species and were used for the first phylogenetic studies of different baculoviruses.

By using monoclonal antibodies in ELISA, it was possible to detect virus antigens in NPV-infected *Helicoverpa armigera* and *Choristoneura fumiferana* larvae at about 6-9 hours after virus exposure, whereas disease symptoms of the larvae could only be observed after 5-6 days (Zhang and Kaupp, 1988; Lu *et al.*, 1995). With an antiserum against the polyhedrin component of the NPV of *Mamestra brassicae*, it was possible to detect polyhedra at a concentration of  $3.13 \times 10^4$  polyhedra/ml by means of immunoelectrophoresis, and as low as  $2.44 \times 10^2$  polyhedra/ml by means of ELISA. Even though this antiserum was specific to MbMNPV, it also cross-reacted with the polyhedrin of *Agrotis segetum* NPV, *Lymantria monocha* NPV and *Neodiprion sertifer* NPV (Riechenbacher and Schliephake, 1988). Similarly, polyhedrin specific antisera were developed for detection of LdMNPV and *Borrelina bombycis* NPV by ELISA in infected host larvae or cultured insect cells (Ma *et al.*, 1984; Shamim *et al.*, 1994). Alternatively, a monoclonal antibody against the 42K protein of AcMNPV was used for virus detection in dead larvae and for safety investigations (Naser and Miltenburger, 1983).

**Figure 4.1 Morphological characteristics of nucleopolyhedroviruses and granuloviruses****B. Biological characteristics*****Host range***

Host range and cross infectivity of many baculoviruses have been reviewed by Gröner (1986). The infectivity of NPV and GV to alternate hosts was typically evaluated on basis of virus infection and mortality of test larvae after oral virus application. However, these examinations are biased toward Lepidopteran species and economically important insects. So far, a standardisation of bioassays to determine host range and specificity is lacking (Cory *et al.*, 1997).

***Nucleopolyhedroviruses (NPV)***

NPV are widely distributed among more than 400 arthropod species belonging to seven insect orders, which are Lepidoptera, Hymenoptera, Diptera, Coleoptera, Thysanura, and Trichoptera, as well as from Decapoda (class Crustacea) (Murphy *et al.*, 1995). In general, the host range of most NPV is restricted to one or a few species of the genus or family of the host where they were originally isolated. Some of the few exceptions having a broader host range are (i) AcMNPV infecting more than 30 species from about 10 insect families, all within the order Lepidoptera, (ii) *Anagrapha falcifera* NPV infecting more than 31 species of Lepidoptera from 10 families and (iii) MbMNPV which was found to infect 32 out of 66 tested Lepidopteran species from 4 different families (Gröner, 1986; Doyle *et al.*, 1990; Hostetter and Puttler, 1991).

### *Granuloviruses (GV)*

GV infections have been reported for more than 100 insect species, however they appear to infect only members of the order Lepidoptera (Murphy *et al.*, 1995). In contrast to NPV, the host range of GV appears to be even more narrow and mostly restricted to a single species. (see also C 23.3)

#### ***Gross pathology***

##### *Nucleopolyhedroviruses*

The gross pathology post infection (p.i.) of NPV infecting Lepidopteran larvae can be summarised as follows:

- Day 1 - 3 p. i.: Infected larvae normally do not show obvious signs of disease.
- Day 4 - 6 p. i.: Diseased larvae only react slowly to tactile stimuli. The larvae start to appear swollen, glossy and moribund.
- Day 6 - 7 p. i.: Diseased larvae stop feeding and begin to die. Diseased larvae of some species, *e.g. Lymantria spec.*, crawl to the top of the twigs (negative geotropism) on which they were feeding.
- Day 7-10 p. i.: Diseased larvae die and may liquefy, the cuticle ruptures and polyhedra are released.

##### *Granuloviruses*

In general three different types of gross pathology with GV can be distinguished (Federici, 1997).

- Many GV such as CpGV show a similar gross pathology in infected larvae as described for NPV.
- Some GV, esp. many Noctuid-infecting GV also pass the midgut epithelium but then infect only fat body tissue. Infected larvae do not stop feeding and grow even bigger than healthy larvae. Larval death occurs at 10-15 days.
- The third type of gross pathology has to date only been observed with *Harrisina brillians* GV. Infection is restricted to the midgut epithelium which causes heavy diarrhetic disorder and death within 4 to 7 days.

#### **C. Pathological characteristics**

Baculovirus pathogenesis has been most extensively studied for its type species AcMNPV, but appears to be similar in all other known baculoviruses (see below).

Most typically, virus replication of NPV occurs in the nuclei of infected host cells, whereas in GV-infected cells the nuclear membrane disrupts during the replication process and loses its integrity. Upon infection, the nuclei appear to become hypertrophic. The occlusion bodies produced by infected cells can be detected by light microscopy.

Divergent tissue tropism is observed with different viruses in their respective hosts. Most NPV specific for lepidopteran species as well as most GV establish a transient infection of the midgut epithelium and then invade other tissues such as fat body, epidermis, tracheal matrix, muscle, nerve, malpighian tubules, and reproductive and glandular tissues. In contrast, NPV specific for Hymenoptera, most Diptera, Trichoptera, Thysanura, and Crustacea as well as the *Harrisina brillians* GV were only found to infect midgut epithelium cells but not any other larval tissue (Federici, 1997).

#### **D. Genetic characteristics**

##### ***Restriction mapping***

Early studies of the size and GV content of baculovirus DNA were based on a number of different methods, such as electron microscopy, melting point determination and reassociation kinetics, ultra-centrifugation in sucrose gradients and chemical analyses.

Restriction endonuclease analysis of isolated DNA has been for many years one of the most important and powerful tools for characterisation and identification of DNA viruses including baculoviruses (Rohrmann *et al.*, 1978). This method allows investigators (i) to identify unequivocally the infecting agent, *e.g.* when a latent baculovirus is activated (Jurcovicova, 1979), (ii) to distinguish among different viruses or virus isolates, which infect the same host species and show similar biological properties (Vlak, 1980), and (iii) to recognise the identity of a virus infecting different host species (Miller and Dawes, 1978). Furthermore, restriction endonuclease analysis is indispensable for the construction of physical maps, which assign the position of the restriction fragments to each other. By convention, the smallest restriction fragment containing the gene encoding polyhedrin or granulin was chosen as the zero point of baculovirus physical maps (Vlak and Smith, 1982). To date restriction maps have been constructed for 18 NPV and 5 GV (Table 4.2).

Table 4.2 Restriction maps of different nucleopolyhedroviruses and granuloviruses

Virus species	References
<b>Nucleopolyhedroviruses</b>	
AcMNPV	Miller and Dawes, 1979; Smith and Summers, 1979; and 1980; Cochran <i>et al.</i> , 1982; Brown <i>et al.</i> , 1984
AgMNPV	Vlak, Johnson and Maruniak, 1989
<i>Amsacta albistriga</i> NPV	Anuradha <i>et al.</i> , 1995
<i>Anagrapha falcifera</i> NPV	Chen <i>et al.</i> , 1996
BmNPV	Maeda and Majima, 1990
<i>Buzura suppressaria</i> SNPV	Qi and Huang, 1987; Liu <i>et al.</i> , 1993
CfMNPV	Arif <i>et al.</i> , 1984
HzenSNPV	Knell and Summers, 1984
<i>Helicoverpa</i> (= <i>Heliiothis</i> ) <i>armigera</i> NPV	Jin and Cai, 1987
LdMNPV	Smith <i>et al.</i> , 1988; McClintock and Dougherty, 1988
MbMNPV	Possee and Kelly, 1988
OpMNPV	Chen <i>et al.</i> , 1988
<i>Panolis flammea</i> NPV	Possee and Kelly, 1988; Weitzmann <i>et al.</i> , 1992
SeMNPV	Wieggers and Vlak, 1984; Heldens <i>et al.</i> , 1996
SfMNPV	Maruniak <i>et al.</i> , 1984
<i>Spodoptera littoralis</i> NPV	Croizier <i>et al.</i> , 1989
<i>Spodoptera litura</i> NPV	Meizhen and Yiquan, 1990
<b>Granuloviruses</b>	
ArGV	Smith and Crook, 1988
CpGV	Crook <i>et al.</i> , 1985; Crook <i>et al.</i> , 1997
<i>Cryptophlebia leucotreta</i> GV	Jehle <i>et al.</i> , 1992
TnGV	Hashimoto <i>et al.</i> , 1996
<i>Xestia c-nigrum</i> GV	Goto <i>et al.</i> , 1992

### DNA homology

Early homology studies of baculovirus strains have been accomplished by comparative restriction analysis and by the estimation of sequence relationships from the number of co-migrating restriction fragments in agarose gels by using the formula of Upholdt (1977). However, this method is only useful for closely related virus strains with more than 90% sequence homology. It does not take into

consideration that many genomic variations are due to small sequence insertions and deletions which result in differences of the restriction profiles even of very closely related virus strains.

DNA hybridisation techniques, such as Southern blot, dot blot and cross blot analysis, have been widely applied for identification and quantification of the intergenomic relationship of many baculoviruses. These methods permit the identification of heteroduplex formation of two different DNAs if they show more than 67% sequence identity (low stringency) or more than 85% identity (high stringency) (Howley *et al.*, 1979).

Smith and Summers (1982) analysed the genomic interrelationship of 18 baculoviruses by restriction analysis, Southern hybridisation and semi-quantitative dot blot hybridisation. They found that under low stringency conditions all viral DNAs showed detectable cross hybridisation. Their results also corroborated the earlier classification of baculoviruses into three subgroups (nuclear polyhedrosis viruses, granulosis viruses and non-occluded baculoviruses, see A 1.1).

The combination of physical mapping and cross hybridisation indicated that the genomes of baculoviruses are similarly arranged. Leisy *et al.* (1984) showed that OpMNPV and AcMNPV are two distinct viruses with a colinear genomic arrangement. Similar observations were made for the genomes of CfMNPV and AcMNPV (Arif *et al.*, 1984), MbMNPV and *Panolis flammea* NPV (Possee and Kelly, 1988) as well as for ArGV and CpGV (Crook *et al.*, 1997) and for *Cryptophlebia leucotreta* GV and CpGV (Jehle *et al.*, 1992).

### 3. Information on the Recipient Organism's Reproductive Cycle

#### A. In vivo and in vitro replication of baculovirus in permissive hosts

##### *Initial stages of infection*

The replication of AcMNPV has been most extensively studied in larvae of *Trichoplusia ni* and in cultured cells of *Spodoptera frugiperda* and serves as a model for NPV and GV replication in Lepidoptera (reviewed by Granados and Williams, 1986; Federici, 1997; Williams and Faulkner, 1997).

The natural route of infection is the peroral ingestion of viral occlusion bodies by larvae. In the alkaline environment of the midgut (pH > 9.5), the occlusion bodies dissolve rapidly and occlusion-derived virions (ODVs) are released. There is evidence that the dissolution of the occlusion body matrix might be facilitated by an insect derived alkaline protease which is associated with the occlusion body matrix. The ODVs pass through the peritrophic membrane (PM), a proteinaceous-chitinous layer which is secreted by the midgut cells to protect the midgut epithelium from direct contact with ingested material. After attachment to the microvilli of the midgut epithelium, the nucleocapsids enter the cell lumen either via fusion of the virion envelope with the epithelial membrane or by viropexis. The nucleocapsids are transported, most likely under involvement of the cellular microtubular structures, to the nucleus and become uncoated at the nuclear pore or within the nucleus where the viral DNA is released and DNA expression and replication is initiated.

##### *The different temporal phases of gene expression*

The following stages and secondary infections initiated by budded virions (BV) of host tissues or cultured cells are thought to be similar. In the early stages (8 hr p. i.) of infection the nucleus becomes hypertrophic and a virogenic stroma is formed where DNA replication and nucleocapsid assembly take place. Host cell protein synthesis is completely shut off by 24 hr p.i.

Viral gene transcription and expression follows a temporally co-ordinated cascade. Early and delayed early genes ( $\alpha$  and  $\beta$  genes) are transcribed by a host dependent RNA polymerase II, which is sensitive to alpha-amanitin. These genes are necessary for regulation of viral gene transcription, for viral DNA replication, and late gene expression. Their promoters resemble those of host genes in having a CAGT transcription initiation site which is 25-31 bp downstream of TATA box and are recognized by nuclear extracts of uninfected cells.

Transcription and expression of late genes (or  $\gamma$  genes) involved in DNA replication, production of structural proteins and budded virions (BV) occurs between 8 and 24 hr p.i.

These genes as well as the very late (or  $\delta$  genes) (see below) have a universal and invariant (A/G/T)TAAG transcription initiation site and are transcribed by a viral encoded RNA polymerase insensitive to alpha-amanitin. Five essential (*p143*, *ie-1*, *lef-1*, *lef-2*, and *lef-3*) and five stimulatory genes (*dnapol*, *p35*, *ie-2*, *lef7*, and *pe38*) were identified to be involved in AcMNPV DNA replication. In addition, transient expression studies using the chloramphenicol acetyl transferase (*cat*) reporter gene under the control of the late and very late promoters (*vp39*, *p6.9*, *polyhedrin*, *p10*) showed that eighteen AcMNPV genes (those necessary for DNA replication and *lef-4*, *lef-5*, *lef-6*, *lef-8*, *lef-9*, *lef-10*, *lef-11*, *p47*, and 39K) were essential for optimal expression from these promoters (Lu and Miller, 1995a). DNA replication and late viral gene transcription can be blocked by aphidicolin (Miller *et al.*, 1981; Rice and Miller, 1986).

Expression of very late genes (or  $\delta$  genes) begins about 18 - 24 hr p.i. and is characterised by a dramatic increase of *polh* and *p10* transcription and expression. *Polh* encodes the polyhedrin (its homologue in granuloviruses is called granulins gene), which is the major component of the occlusion body matrix. The *p10* gene product is associated with the formation of extensive fibrillar structures in the nucleus and cytoplasm of infected cells (Quant-Russell *et al.*, 1987; van der Wilk, *et al.*, 1987). Although the biological function of *p10* has not been convincingly elucidated so far, it was suggested that it might contribute to the disruption of the nuclear membrane and to the release of occlusion bodies from infected cells (van Oers *et al.*, 1993; Williams *et al.*, 1989).

Since both *polh* and *p10* are not required for DNA replication and are hyperexpressed during cell infection, their strong promoters were exploited for the development of the baculovirus expression vector systems. The coding region of these genes can be replaced by exogenous genes resulting in the production of high levels of the foreign protein (Smith *et al.*, 1983; Vlak *et al.*, 1990).

#### *Production of budded viruses (BVs) and occlusion derived viruses (ODVs)*

The replicative cycle of baculoviruses is biphasic and generates two distinct viral phenotypes, the budded virus (BV) and the occlusion derived virus (ODV). The two phenotypes are structurally distinct and destined for two different functions, both of which are essential for virus survival in nature. ODVs are released from the inclusion bodies and infect midgut epithelium cells, where a first round of virus replication takes place. The newly produced nucleocapsids traverse the nuclear membrane, the cytosol and bud through the basal lamina of the midgut cells into the hemolymph. These budded virions (BV) acquire a new envelope which consists of plasma membrane containing peplomers of a viral encoded glycoprotein, termed gp64 (see Fig. 1 in A 2.1). Gp64 appears to be pivotal for the interaction between the BV envelope and susceptible host cells through a possible interaction with a cell membrane receptor molecule and then a final fusion with the endosomal membrane (for review see Blissard, 1996). In cultured cells the production of BV peaks during the late phase of gene expression, between 10 - 20 hr p.i., whereas the very late occlusion phase can be observed between 16 - 72 hr p.i.

For most NPV and GV infecting lepidopteran host larvae, virus occlusion is not observed in midgut epithelial cells. These cells release BV into the hemolymph which then systemically spreads the virus infection among susceptible cells and tissues. In contrast, NPV of Hymenoptera, Diptera and Crustacea and the *H. brillians* GV infect only midgut cells where occluded viruses are produced (for review see Federici, 1997). However, there is also evidence that some nucleocapsids might traverse the midgut epithelial cells without replication and bud directly into the hemolymph (Granados and Lawler, 1981).

Engelhard *et al.* (1994) used a recombinant AcMNPV mutant expressing *lacZ* reporter gene (beta-galactosidase) to study the infection pathway in fourth instars of *Trichoplusia ni*. Based on the observation of early infection of midgut tracheoblast and the tracheal matrix, they postulated that the tracheol system might directly contribute to the systemic spread of BV. This finding, however, is not supported by others who used a similar approach (Flipsen *et al.*, 1993; 1995).

Altogether, the spread of BV and systemic infection starts from the midgut and continues to hemocytes, tracheal cells, fat body, muscle and nerve cells as well as reproductive and glandular tissues. In the final step of infection, occlusion bodies are formed and the nuclei are packed with occlusion bodies which causes the cellular hypertrophy and swollen appearance of the infected larvae.

The different role of BVs and ODVs in the reproduction of baculoviruses can be summarised as follows: ODVs transmit infection from one larvae to another within an insect population, whereas the budded virus (BV) spreads the infection within susceptible larval tissues.

## B. Behaviour in semi- and non-permissive insect cells

Baculovirus gene expression, replication and reproduction is only possible if one of the virion phenotypes (BV or ODV) is able to enter a permissive host cell. There are a number of viral factors and host responses which are necessary for a productive infection. In a non-compatible baculovirus-host cell interaction, the baculovirus gene expression and replication is blocked at a early stage. In order to analyse the genetic factors involved in host specificity and baculovirus-host cell interaction, a number of studies on the effect of virus on semi- and non-permissive insect cell lines were conducted. Infection studies with vertebrate and mammalian cells will be discussed in Section B.

When *T. ni* cells (TN-368) were infected with SfMNPV, typical early cytopathological effects such as nuclear hypertrophy and the formation of a virogenic stroma could be observed. However, only a few early transcripts and two proteins, an early 97 kD and a late 29 kD polypeptide, were synthesised. Virion assembly could not be detected. This result suggested that the block of infection occurred during the early phase of gene expression (Carpenter and Bilimoria, 1983; Bilimoria, 1991).

AcMNPV infection of *Choristoneura fumiferana* cell lines Cf124T and CF-203 is blocked after DNA replication and causes apoptotic cell death, respectively (Liu and Carstens, 1993; Palli *et al.*, 1996). An apoptotic response to AcMNPV infection has also been observed for *Spodoptera littoralis* cell line SL2 (Chejanovsky and Gershburg, 1995).

By using recombinant AcMNPV expressing chloramphenicol acetyltransferase (*cat*) reporter gene under control of different temporally regulated promoters such as early, late and very late promoters, Morris and Miller (1992; 1993) showed that the activity of late and very late promoters is significantly reduced in semi- and nonpermissive cell lines of *Bombyx mori* (BmN-4), *Choristoneura fumiferana* (CF-1), *Lymantria dispar* (Ld652Y), *Mamestra brassicae* (MaBr-3) and *Drosophila melanogaster* (Dm) compared to permissive cell lines of *Spodoptera frugiperda* (Sf-21). Since there was some evidence for viral DNA replication but not for virus assembly, it was suggested that in these

cell lines progression of infection is blocked during DNA replication. By infecting different cell lines with AcMNPV expressing *cat* reporter gene controlled by the Rous sarcoma terminal repeat promoter and  $\beta$ -galactosidase controlled by the very late polyhedrin promoter, it was shown that nonpermissive *Drosophila* cells and permissive Sf-21 had a similar *cat* expression, whereas very late promoter activity was only observed with Sf-21 (Carbonell *et al.*, 1985, Carbonell and Miller, 1987).

### C. Genes involved in host range determination

So far, a number of baculovirus genes involved in differential host cell and host larval specificity have been identified. There are several examples where the expression or deletion of these host range determinants by recombinant NPV allowed the specific extension or restriction of host specificity. These results, mainly obtained with AcMNPV, provide evidence that a baculovirus which is infective to different host species relies on specific genes to establish infection and virus replication and that these sets of genes might differ slightly from host species to host species.

Although genetically closely related, AcMNPV and BmNPV have distinct host range specificities. Kondo and Maeda (1991) demonstrated that an AcMNPV mutant containing part of the BmNPV *p143* gene became infective to the normally refractile BmN cell line of *Bombyx mori*. This mutant, called eh2-AcMNPV, was obtained after coinfection of Sf-21 cells with AcMNPV (OT2) and BmNPV(T3), which are not infective for BmN and Sf21 cells, respectively. The progeny virus was subsequently passaged through BmN and Sf-21 cells thereby isolating eh2-AcMNPV which was found to be infective for both cell lines. In further experiments, the genome region responsible for host range extension was more precisely localised on a 572-bp fragment of BmNPV *p143*, which differed in 14 out of 109 amino acids as compared to AcMNPV (OT2) (Maeda *et al.*, 1993). Similar results were obtained by Mori *et al.* (1992) who performed the co-transfection experiments with fragments of the BmNPV genome instead of BmNPV virions. This finding was corroborated by studies of Croizier *et al.* (1994) who demonstrated that the exchange of only three amino acids within *p143* was sufficient to expand the host range of AcMNPV to *B. mori* cells. It appears that the substitution of a single serine residue to an asparagine residue of AcMNPV *p143* is sufficient for this host range extension (Kamita and Maeda, 1997).

Infection of the *Lymantria dispar* cell line Ld652Y with AcMNPV is characterised by early cytopathic effects, transcription from all temporal classes of promoters and DNA replication but with a very low level of protein translation and no formation of infectious virions (McClintock *et al.*, 1986; Guzo *et al.*, 1991; 1992). However, when Ld625Y cells were coinfecting with AcMNPV and LdMNPV, replication and production of AcMNPV was observed suggesting that LdMNPV encodes a trans-acting factor which rescues an abortive AcMNPV infection (McClintock and Dougherty, 1987). This trans-acting factor encoded by LdMNPV was finally mapped and identified by co-transfecting Ld625Y cells with AcMNPV genomic DNA and single cosmids of the LdMNPV genome. It was called *host range factor 1* (*hrf-1*) and was shown to extend the host range of recombinant AcMNPV expressing the factor to Ld652Y cells and to *L. dispar* larvae. This indicates that *hrf-1* determines the host range at the cell culture and larval levels (Thiem *et al.*, 1996).

Transient expression studies using the *cat* reporter gene revealed that eighteen AcMNPV genes, so-called *lef* genes, were essential for optimal expression of late and very late genes in Sf21 cells (see also Chapter 3.1.2). For the permissive *T. ni* cell line TN-368, it was found that in addition to these 18 *lef* genes an additional gene, called *host cell-specific factor-1* (*hcf-1*) was required for efficient late gene expression. AcMNPV mutants lacking *hcf-1* replicate normally in Sf21 cells and *S. frugiperda* larvae but are unable to productively infect TN-368 cells and *T. ni* larvae (Lu and Miller, 1995b; 1996; reviewed by Miller and Lu, 1997). This observation clearly indicated that *hcf-1* has tissue specific as

well as species-specific effects on the replication of AcMNPV in cultured insect cells and in insect larvae.

Abortive replication in the permissive cell lines SF-21 (and SF-9) was observed with AcMNPV mutants lacking a functional *p35* gene. *p35* is an inhibitor of programmed cell death (apoptosis) of insect cells, which might be a defence reaction of insects against baculovirus infection at the organismal level (Clem *et al.*, 1991; for review Friesen, 1997). It appears that *p35* also exerts host range function since this gene is essential for replication in SF-21-cells and *S. frugiperda* larvae but not in TN-368 and *T. ni* larvae. AcMNPV *p35* mutants were 1000-fold less infective for *S. frugiperda* larvae than wildtype-AcMNPV when the virus is injected into the hemocoel and they were about 25-fold less infective in peroral infections (Clem and Miller, 1993; Clem *et al.*, 1994).

#### 4. Biological Features and Environmental Conditions Which Affect Survival, Reproduction, Growth, Multiplication or Dissemination

##### A. Formation of occlusion body

NPV and GV form polyhedral and granular occlusion bodies, respectively, into which the occlusion derived virions (ODV) are embedded. The occlusion bodies serve to protect the embedded virions against damaging environmental conditions and allow the virions to remain viable for many years. The occlusion bodies are solubilised in alkaline conditions of the midgut and thus deliver the ODV to susceptible columnar epithelial cells.

The crystalline matrix of the occlusion body mainly consists of a single protein, called polyhedrin and granulins, respectively. These proteins of about 245 amino acids (29 kDa) are hyperexpressed during the very late phase of virus infection and are not required for virus replication (for review see Rohrmann, 1992; Funk *et al.*, 1997). To date, polyhedrin and granulins genes of about 35 different lepidopteran- and one hymenopteran-specific baculovirus species have been sequenced. The polyhedrin and granulins genes are highly conserved with at least 70% amino acid identity among lepidopteran NPV and about 50% amino acid sequence identity between NPV polyhedrins and GV granulins.

The importance of the occlusion body for the stability and maintenance of infectivity of baculoviruses in the environment has been clearly demonstrated by field tests using polyhedrin deficient AcMNPV mutants. In 1987, a field test was performed where insect larvae were infected with an AcMNPV polyhedrin minus mutant and then released into enclosed field plots. The non-occluded virus progeny rapidly lost its activity in the decaying larval carcass and no virus activity could be detected on cabbage leaves or in soil samples within two weeks after the release (Bishop *et al.*, 1988a; 1988b). Wood and co-workers applied a slightly different approach by co-occluding polyhedrin-minus AcMNPV into the polyhedrin matrix of wild-type virus (Hamblin *et al.*, 1990). In laboratory experiments it was shown that the persistence of such a co-occluded polyhedrin-minus mutant was significantly reduced at inoculum levels below a 100% dose. This observation was corroborated in a three-year field test in which AcMNPV polyhedra containing polyhedrin minus and wild-type AcMNPV in a ratio of 48:52 were applied on *T. ni* larvae, and the amount of polyhedrin minus mutants was analysed in the following years. It was found that the amount of polyhedrin minus mutant dropped below 20% of the virus progeny in the second and third year (Hamblin *et al.*, 1990; Wood *et al.*, 1994).

The occlusion bodies are not solely composed of polyhedrin. They are surrounded by an envelope, called a polyhedron calyx or polyhedron envelope (PE). Minion *et al.* (1979) reported that the PE of *Helicoverpa* (= *Heliothis*) *virescens* NPV was composed of hexose and pentose

carbohydrates. Whitt and Mannig (1988) showed that the PE of AcMNPV consisted of a phosphorylated protein which might be covalently linked to the carbohydrate component. The PE protein is encoded by a late and very late gene (*pp34*) which has been sequenced for several NPV (Gombart *et al.*, 1989; Bjornson and Rohrmann, 1992). OpMNPV mutants lacking the PE gene produced unstable polyhedra with a rugged and pitted surface (Gross *et al.*, 1994a).

Another protein which is normally associated with polyhedra in the infected cells is the *p10* protein. Evidence suggests that it is involved in the formation of PE and lysis of infected nuclei. AcMNPV mutants with inactivated *p10* genes failed to release the polyhedra from infected cells. *p10* negative OpMNPV and AcMNPV mutants also failed to form an intact polyhedron envelope generating fragile polyhedra with significantly reduced stability but with the peculiarity to form polyhedral aggregates (Williams *et al.*, 1989; van Oers *et al.*, 1993). Based on these observations, it was suggested that the function of *p10* and PE is twofold: to protect the polyhedra from mechanical damage by sealing their surface and to prevent their aggregation. Hence, these properties could be important to maximise the number of intact virions per occlusion body and to optimise virus dissemination (Gross *et al.*, 1994a).

### **B. Enhancing of host susceptibility**

Synergism between *Pseudaletia unipuncta* NPV and GV, which resulted in an increased susceptibility of *P. unipuncta* larvae for the NPV, has been described (Tanada, 1959). A protein component in the granule of PsunGV, termed synergistic factor, which interacts with the microvillar membrane of midgut cells and facilitates adsorption of the NPV virions was identified (Tanada *et al.*, 1975; Zhu *et al.*, 1989).

Similar factors have been found in TnGV, *Xestia c-nigrum* GV and *Helicoverpa* (= *Heliothis*) *armigera* GV (Derkens and Granados, 1988; Goto, 1990; Roelvink *et al.*, 1995). These proteins are commonly known as enhancing factors or enhancin. TnGV enhancin was shown to increase the infectivity of AcMNPV to different noctuid larvae. The predicted amino acid sequences of TnGV enhancin and the synergistic factor of PSunGV-H are almost identical. TnGV enhancin is a protein of 104 kDa which most probably functions as a metalloprotease, since it can be reversibly inactivated by metal chelators (Lepore *et al.*, 1996). TnGV enhancin is located in the occlusion body and causes specific degradation of the intestinal mucin component of the peritrophic membrane (Wang and Granados, 1997). The disruption of the peritrophic membrane of the larval midgut facilitates the virions access to the midgut columnar cells.

Although such a function was also found with a component of NPV polyhedra, no gene homologue to the TnGV enhancin could be identified in NPV (Derkens and Granados, 1988; Hashimoto *et al.*, 1991). Observations of the synergistic enhancement of infectivity by the activity of components from different NPV, and the isolation of another synergistic factor from *Pseudaletia unipuncta* GV indicate that a somewhat heterogeneous set of genes and functions may contribute to this modulation of the infectious process (Arne and Nordin, 1995; Ding *et al.*, 1995).

### **C. Inhibition of cellular apoptosis**

Programmed cell death or apoptosis is a cellular pathway during which a cascade of responses is activated resulting in a well regulated cellular suicide. Apoptosis is the normal fate of many cells during development and metamorphosis, cellular turnover of renewing tissues and other dynamic cellular processes. There are also many examples of viral infections whose progression is blocked during early stages of replication by an apoptotic response of the infected host cell. It appears that apoptosis is a very powerful mechanism of many vertebrate and invertebrate cells to prevent viruses

from replicating and becoming persistent. On the other hand, viruses have evolved mechanisms that overcome or block this apoptotic response and so establish an infection (for review see Clem, 1997).

In baculoviruses two classes of proteins, the *p35* protein and the IAP (inhibitor of apoptosis) proteins, have been identified and characterised as anti-apoptotic agents. Expression of *p35* was found to be essential for AcMNPV replication in *S. frugiperda* cells (Clem *et al.*, 1991). SF-21 cells infected with an AcMNPV ‘annihilator’ mutant, which was shown to contain a deletion in *p35* gene, underwent apoptotic cell death within 24 hours. The antiapoptotic effect of *p35* has been demonstrated not only in insect cells but also in many heterologous systems, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, mammalian and human cells (Sugimoto *et al.*, 1994; Hay *et al.*, 1994; Rabizadeh *et al.*, 1993; Beidler *et al.*, 1995). The competence of *p35* to prevent apoptosis in many different cell types pointed to the possibility that *p35* acts on a universal step in the apoptotic cascade. Xue and Horvitz (1995) and Bump *et al.* (1995) demonstrated that *p35* is able to inhibit cysteine proteases, so-called ICE-like proteases, which play a key role in the highly conserved effector pathway of apoptotic response.

Compared to wild-type viruses, the infectivity of *p35* minus AcMNPV mutants to *S. frugiperda* larvae was dramatically reduced (by a factor of 1000 if injected into the hemocoel and by a factor of 25 if perorally applied). The yield of virus progeny from infected larvae was decreased 900-fold suggesting that the anti-apoptotic effect of *p35* may play an important role during *in vivo* infection of host larvae. In contrast to SF-21 cells, *p35* is not essential for AcMNPV to establish infection in TN-368 nor is there any difference between *p35* minus and wildtype AcMNPV in the infectivity for *T. ni* larvae. This suggests that *p35* is also involved in determining a baculovirus host range (Clem and Miller, 1993; Clem *et al.*, 1994). A *p35* homologue has been also identified in BmNPV but is not present in OpMNPV (Maeda, 1994; Ahrens *et al.*, 1997).

A second class of antiapoptotic genes of baculoviruses are *iap* genes, which were identified in CpGV and OpMNPV because of their ability to rescue replication and polyhedra formation of *p35* minus AcMNPV mutants in SF-21 cells (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). All baculovirus *iap* proteins contain two cysteine-rich repeats known as BIR (baculovirus *iap* repeat) at their N-termini and a RING finger motif at the C-terminus. Further *iap* genes were identified upon genome sequencing in AcMNPV (*iap1*, *iap2*), BmNPV (*iap1*, *iap2*) and OpMNPV (*iap1-iap4*) (Ayes *et al.*, 1994; Maeda, 1994; Ahrens *et al.*, 1997). Cellular *iap* homologues were identified in humans and insects and might be ubiquitous in a wide variety of organisms (for review see Clem, 1997). Although the mode of action of *iap* is not well understood, there is strong evidence that both *iap* and *p35* inhibit defence mechanisms at the cellular and organismal levels, thus allowing baculoviruses to replicate and reproduce.

#### D. Arresting host development by ecdysteroid-UDP-glucosyltransferase

One of the most remarkable features of baculoviruses is their ability to impair larval moulting. In normal development, larval moulting is a physiological process regulated by ecdysteroid hormones. It was found that AcMNPV is able to inhibit larval moulting by expressing the enzyme ecdysteroid-UDP-glucosyltransferase (EGT). EGT inactivates insect ecdysteroid hormones by conjugation with glucose or galactose residues (O'Reilly and Miller, 1989). Whereas, the moulting process in healthy larvae is characterised by a cessation of feeding, baculovirus infected larvae do not show this behaviour. By generating an EGT minus AcMNPV mutant and comparing its pathogenicity with wild-type virus, O'Reilly and Miller (1989) found that EGT expression is responsible for the suppression of the host development during infection. Molecular studies showed that EGT is transcribed and expressed early during infection (O'Reilly and Miller, 1990). To date, homologous EGT genes have

been identified and sequenced from 12 nucleopolyhedroviruses and it seems very likely that it is a conserved ancestral gene present in all baculoviruses (Chen *et al.*, 1997).

Since EGT minus AcMNPV mutants yielded about one fourth less virus progeny in late *S. frugiperda* instars than wild-type AcMNPV, it was proposed that EGT expression might have an important role in maximising the amount of virus progeny from an infected larva (O'Reilly and Miller, 1991, for review see O'Reilly, 1997). A further intriguing observation was that *egt* minus AcMNPV mutants killed host larvae faster than wild-type virus. The mean survival time (ST<sub>50</sub>) of EGT minus AcMNPV-infected *S. frugiperda* and *T. ni* larvae was about 20-30% shorter than that of wild-type AcMNPV (O'Reilly and Miller, 1991; Eldridge *et al.*, 1992). Although the reason for this increased virulence is not yet elucidated, there is apparently a correlation with the decreased amount of virus progeny. In the faster dying insects, the virus does not have enough time to produce the maximum amount of virus progeny.

The accelerated mortality of the EGT minus AcMNPV mutant accompanied by reduced feeding of the infected larvae led to a new concept of genetically engineered baculoviruses with improved insecticidal properties (O'Reilly and Miller, 1991). In recent years, EGT minus baculoviruses have been tested in the field and they are the most likely candidates to become the first registered genetically engineered baculovirus control agents.

#### **E. Facilitating the release of occlusion bodies from larval cadavers**

The final step of baculovirus infection is the breakdown of the larval cuticle and the release of the occlusion bodies into the environment. The cuticles of insect larvae consist mainly of chitin fibres embedded into a proteinaceous matrix. Two baculovirus genes, encoding a chitinase (*chiA*) and cathepsin (*cath*), have been described to contribute to the liquefaction of the larval carcass and the release of occlusion bodies. Chitinase is a chitin-degrading enzyme with endo- and exomolecular specificity, whereas cathepsin has cysteine proteinase activity (Slack *et al.*, 1995; Hawtin *et al.*, 1995).

The *chiA* and *cath* genes appear to be located next to each other in many baculovirus genomes and are expressed late in infection (for review see O'Reilly, 1997). The functional role of *chiA* and *cath* was elucidated by constructing AcMNPV and BmNPV mutants lacking either *chiA* or *cath* or both. Both single *chiA*- or *cath*-minus mutants were not able to cause liquefaction of infected larvae (Slack *et al.*, 1995; Hawtin *et al.*, 1995). Hence, the co-operative action of chitinase and cathepsin appears to exert an effect on the release of viruses from succumbed larvae and to facilitate the dissemination of occlusion bodies in the environment.

An additional role of *cath* was proposed by Lanier *et al.* (1996) who found that cathepsin is associated with BV of AcMNPV and is able to cleave actin in an *in vitro* assay. Whether cathepsin is also involved in the rearrangement of actin filaments during early stages of infection needs further investigation

#### **F. Latent virus infections**

Latency is the ability of a virus to persist in a host without causing disease symptoms, thereby providing the virus the possibility of vertical transmission from one generation to another. There are a number of early publications reporting circumstantial evidence of latent baculovirus infections. UV light and treatment with chemicals, rearing conditions, or superinfection with a different virus have been described as reasons for activating latent infections (Krieg, 1956; David and Gardiner, 1965; Longworth and Cunningham, 1968; Biever and Wilkinson, 1978; Podgwaite and Mazzone, 1986). However, most of these early studies were made when molecular tools for identification such as

hybridisation techniques or restriction endonuclease analysis were not available. Hence, it cannot be excluded that at least some of these reports were biased by cross-infections and contamination. Ponsen and de Jong (1964) and Jurcovicova (1979) reported activation of latent *Adoxophyes orana* SNPV by infecting *A. orana* larvae with *Barathra brassicae* MNPV and vice versa activation of latent *B. brassicae* MNPV by infection of *B. brassicae* larvae with *A. orana* SNPV. An increase in larval deaths from granulovirus infection correlated to stress from dehydrated diet or low temperature was observed in *Pieris rapae* (Biever and Wilkinson, 1978). Virulence testing indicated the identity of the stress induced virus with laboratory grown virus preparations. It was noted that larvae demonstrating symptoms of stress induced virus infection could recover to a healthy condition after relief of the stress by providing a fresh diet.

More recently, Hughes *et al.* (1993) provided first molecular evidence for activation of a latent baculovirus in *M. brassicae*. Activation of a MbMNPV was observed after feeding *Panolis flammea* NPV or AcMNPV to the laboratory culture of *M. brassicae* termed MbLC. Other (environmental) stress factors such as high and low temperatures, starvation or crowding were not effective in triggering apparent infections in this culture (Goulson unpublished data, Goulson and Cory, 1995a). Contamination of the virus inoculum was excluded by control infections of another *M. brassicae* culture (MbWS). Challenging this insect culture, established from a novel environmental isolate and adapted to laboratory growth conditions, did not result in multiplication of MbMNPV when infected with the same inoculum. Furthermore, MbMNPV-specific sequences were detected by PCR in each stage of insect development, *i.e.* in eggs, larvae, pupae and adults. When dissected larval tissues were analysed, latent virus sequences were only detected in the fat body of MbLC larvae. Cell lines established from fat body cells also harboured MbMNPV-specific sequences.

The occult state of the virus in the MbLC culture was further characterised by analysis of m-RNA in larvae, demonstrating the presence of polyhedrin specific m-RNA. Assays of transient reporter gene expression (CAT, chloramphenicol acetyl transferase) after transfection of primary cultures of MbLC fat body cells with constructs containing early, late and very late promoters, demonstrated the presence of expression factors for all of these, albeit at low levels. Furthermore, inoculation of MbWS larvae with MbLV fat body cells resulting in larval deaths from MbMNPV infection, strongly suggested the presence of viable virus particles in these cells (Hughes *et al.*, 1997). The latent or occult status of MbMNPV in the insect can thus be described as a persistent infection, with a continuous production of virus proteins at a low level. Whether this state of the virus is controlled by host functions exclusively or to which degree viral regulatory functions are engaged in its establishment and support, remains an intriguing and challenging question.

A more detailed knowledge of the possibility and extent of virus persistence as a latent infection with vertical transmission to insect progeny and the potential to induce an infectious cycle through environmental conditions or stress factors, is crucial for an improved understanding of baculovirus ecology and population dynamics. So far, the contribution of this feature to the persistence of baculoviruses in natural populations, and its occurrence among insect and virus species and genotypes, is largely unknown. The perception of baculovirus population dynamics as, for example, represented in the modelling of natural and induced epizootics, almost exclusively considers the multiplication of viruses as a result of infections initiated by horizontal virus transmission.

### G. Environmental factors influencing virus persistence

Virus persistence is an important factor affecting the potential of baculoviruses to interact with their hosts in the environment. Biotic and abiotic persistence mechanisms allow the virus to overcome situations of varying host density without running the risk of extinction (Evans, 1986). Baculoviruses may persist in the host population through vertical transmission of disease or by a latent infection (see

A 4.6) or in non-target organisms which may serve as vectors of virus dispersal (see C 26.2). This biotic persistence complements the maintenance of virus activity outside living organisms (abiotic persistence). In the open environment UV radiation of the sunlight has the most significant impact on abiotic persistence which also may be influenced by temperature, relative humidity and precipitation (for review, see Jacques, 1975). Half life time may be as short as 1.3 days for a purified preparation of the *Phthorimaea operculella* granulovirus (Kroschel *et al.*, 1996) or, more typically, about 5 - 10 days for many NPV and GV (Bell and Hayes, 1994; Kolodny-Hirsch *et al.*, 1993). Hence, a variety of formulations and additives have been tested and applied to improve the life time of preparations for pest control applications (Ignoffo *et al.*, 1989; Ignoffo and Garcia, 1996; see also A.6.3). On the other hand, after spray applications of *Panolis flammea* NPV on pine foliage, only a slight decrease of infectivity was monitored over several months (Carruthers *et al.*, 1988). This observation suggests that the application time of PaflNPV for control of the pine beauty moth, *Panolis flammea*, does not necessarily need a precise optimisation (Cory and Entwistle, 1990). Agricultural operations can affect the distribution of the baculoviruses in soil. Following *A. gemmatilis* NPV spraying on soybeans and subsequent epizootics, it is transported into the soil and decreases rapidly due to overwinter weathering, but enough remains near the soil surface at the beginning of the next growing season to initiate new epizootics. Persistence of the NPV in soil is not adversely affected by disking, cultivating, or other agricultural soil operations (Fuxa and Richter, 1996).

Furthermore, plant surfaces can have a distinct influence on viability and virulence as discussed in section C. 23.4.1. In soil, which can also be a potential reservoir of baculoviruses, the inactivation rate not only depends on soil type and pH but also on microbial activity (Thompson and Scott, 1979; Ignoffo and Garcia, 1966; Jaques and Huston, 1969; Undorf, 1991). Baculoviruses may persist in soil for long periods. Soil samples taken from a field treated with *Trichoplusia ni* NPV showed 15% of the original virus activity 6 years after application (Jaques, 1964). Similar results were obtained for other GVs and NPVs (for review see Jaques, 1975).

In summary, these observations denote that the physical environment, chemical conditions and the (micro)-biological composition of micro-habitats have a very significant impact on the persistence and activity of baculoviruses. The validity of comparisons of the persistence of different virus types depends on the degree of control of experimental variables. In particular cases the determination of the abundance of genotypes in artificial mixtures could be used as an approach. Parameters of persistence used in calculations of virus spread and modelling have either to account for the detailed structure of the respective environment or must include wide margins for the variation of median values. Biotic and abiotic persistence are prerequisites for mechanisms and routes of dispersal (summarised in section C.26).

## **5. Behaviour in Simulated Natural Environment Such as Microcosms, Growth Rooms, Greenhouses, Insectaries, etc**

### **A. Monitoring of baculoviruses in closed environments and ecological modelling**

Experiments such as bioassays (C.23.1) test well defined characteristics of baculovirus phenotypes in the laboratory. The use of microcosms or similar facilities may supplement these investigations by monitoring virus interactions in more complex environments. Microcosms may represent the variables of natural settings to a widely divergent degree, depending on their particular objective. Fraser and Keddy (1997) describe the "manipulation of an individual environmental axis" to explore its function in structuring the community as a common factor of microcosm research in ecology. Research of this kind may contribute to an improved understanding of baculovirus ecology by investigating mechanisms of fate and interactions in the environment in some detail (Cory *et al.*, 1997; Cory and Hails, 1997). Increased knowledge of ecological behaviour will also generate a better

predictability and may improve safety assessments. Perspectives of safety may also trigger microcosm experimentation because some degree of confinement from the open environment is provided.

Traditionally, such testing was not usually performed with natural strains during the development of baculovirus insecticides. Baculovirus behaviour (*e.g.* pathogenicity, virulence, host range, and toxicity for particular non-target organism) is inferred from laboratory testing on one side. On the other, the world-wide application of baculoviruses for pest control together with scientific investigations on baculovirus population dynamics in the open environment contribute to the present understanding of baculovirus ecology. This background of knowledge about the mechanisms driving the dynamics of effects on insect species and about the ability to manipulate it, mostly is considered to be adequate for judgements about applications and safety in the environment. To some extent this view is reflected in the criteria of registration procedures (*e.g.* Andersen *et al.*, 1989).

Conventional testing procedures using soil columns as a section of the natural environment were used to test the leaching behaviour of baculovirus preparations as a component of environmental fate in order to predict potential exposition rates of the groundwater. Rates were tested under conditions similar to pesticide registration procedures (C.26).

The perspective of using genetically modified baculoviruses seems to have changed the perception with respect to testing in "closed" environments to some degree. The first "deliberate release" of a genetically engineered baculovirus can be viewed as an experiment performed in a confined "simulated natural environment" (Bishop *et al.*, 1988). Modified versions of microcosms introduced for environmental transport and fate studies in chemical risk assessment were used in this context to monitor essentially similar parameters of baculovirus fate. Modelsystems of this kind also provided some simulation of meteorological conditions (sunlight, rainfall, and air movement). Investigations included the comparison of modelsystem and field data, and of the persistence and spread of natural and genetically modified virus types. The potential of monitoring individual genotypes was demonstrated to some extent. A reduced spread and multiplication of the modified genotype in some contrast to similar biotest data was noted (Undorf, 1991). However, systematic comparisons of biotest, modelsystem, and field data, in order to analyse their correspondence, are not well documented in the scientific literature. The potentials as well as the limitations of experimentation in an intermediate scale of complexity have not been explored in great detail. This is in contrast to its use for the evaluation and modelling of the fate and effects of chemicals in the environment. The interrelationship of an improved understanding of baculovirus ecology on one side and their biosafety on the other is stressed in recent publications (Cory *et al.*, 1997). Limited relevant modelling supported by experimentation is cited there to exemplify the need to analyse the correspondence between theory, closed environment experimentation and field data for an improved understanding of baculovirus population dynamics (Begon and Bowers, 1994; Begon *et al.*, 1996).

For inferences, empirical data must be interpreted by theoretical structuring of knowledge in some kind of model, representing the essential components and the relations assumed to exist between them. Explicit modelling of baculovirus ecology given a mathematical form tries to calculate the dynamics of viruses and of their arthropod hosts under a given set of parameter variations. Results of such calculations are then compared with empirical data from modelsystem experimentation, field testing, or natural epizootics, to explore their present contribution to an understanding of mechanisms and to a limited degree of predictive potential, eventually. Simulated natural environments have been employed to a limited extent for testing environmental variables, to verify the formulation of interactions, and to analyse parameter values. This type of research is described in the following to account for common experimental approaches and for the correspondence with objectives to improve (predictive) knowledge of baculovirus ecology.

The work of Anderson and May (1980, 1981), cited in reviews of baculovirus ecology with some emphasis, is the key reference for the modelling of the dynamics between pathogens and their invertebrate hosts (Evans, 1986; Cory *et al.*, 1997). In the Anderson and May model, the variations of forest insect pest abundances with low densities between regular extensive population increases were triggered exclusively by the dynamic relations between an insect pest population and pathogens persisting in the environment. Their model assumptions, describing the rate of disease transmission by a mass-action law with a linear relationship between pathogen/host densities and productive infection, have been criticised for incorrectness, oversimplification and lack of correspondence to reality (Bowers *et al.*, 1993; D'Amico *et al.*, 1996; Berryman, 1997). Whereas the statement that "it is no longer possible to claim that mathematical models provide a basis for believing that forest insect cycles might be generated by host-pathogen interactions alone" (Bowers *et al.*, 1993) may be accepted or be viewed as a matter of discussion, the basic structure of the model is further employed as a starting point for modifications and adaptations of parameters and interactions. Cory *et al.* (1997) describe in some detail the modifications of the terms of transmission, virulence, yield, and persistence that are introduced or should be incorporated to generate correspondence with actual knowledge about baculovirus biology, as summarised in different sections of this paper.

Modelsystems, not intending to simulate natural environments to any significant degree, were used by Sait *et al.* (1994) and Begon *et al.* (1996) to study fluctuations in population densities of the Indian meal moth *Plodia interpunctella* including a baculovirus pathogen and a parasite. Systems varied with respect to composition: virus-free populations, infected populations continuously exposed to a granulovirus, or three-species systems including the interaction with the ichneumonid parasitoid *Venturia canescens* were used. A cyclic dynamic of abundance in all systems was recorded, with a very significant dependence of cycle periods upon the composition of the system. The correspondence of the details of the dynamical patterns with predictions from mathematical modelling was assessed only qualitatively.

The variation of viral disease transmission was analysed using different larval instars in laboratory and field studies. Modelling was used to account for different susceptibilities, feeding habits and times of larval death after infection (Goulson *et al.*, 1995). The influence of the parameter of larval population density on transmission of gypsy moth NPV was investigated by D'Amico *et al.* (1996). In order to control the "environmental axis" under investigation, meshes were used to prevent the movement of larvae from their place on red oak foliage. Different numbers of larvae were used to investigate the influence on the transmission coefficient. Its density dependent variability did not correspond to predictions of different modelling approaches, demonstrating the need to redefine this process. A correspondence with data of large scale investigations (Woods and Elkinton, 1987) suggested a common driving force for the type of variability. It was concluded that the large-scale dynamics of the virus may be determined by interactions at the small scale. Further experimentation at a large scale was considered necessary in order to test this hypothesis.

Observations of virus distribution following artificially induced epizootics failed to correspond with any of the predictions included in mathematical modelling of spatial spread (Dwyer and Elkinton, 1995). The mechanism of dispersal in addition to larval movement, including ballooning of first instars, remained a matter of speculation.

In the type of research as described above, field experimentation or observations in the open environment were sometimes used directly to compare model predictions with data. The need for particular data and the convenience of their generation were the main factors determining the level of complexity for investigations. A special role of microcosm experimentation was not appreciated explicitly. Altogether, empirical testing challenges theoretical modelling, to improve its accuracy and

correspondence to reality, in this way increasing its predictive value. In general, the correspondence with data from the open environment is considered the crucial test for the validity of models.

Whereas theoretical investigations have their own merits - in particular by shaping the reflection about essential components and relations, their value for the description of real systems is that of an untested hypothesis. This character compromises the direct applicability for microbial pest control or the prediction of the fate of non-target organisms with a low susceptibility for virus infection (Bowers and Begon, 1991; Begon and Bowers, 1994).

The interrelationship of an improved understanding of baculovirus ecology on one side and their biosafety on the other is stressed in recent publications (Cory *et al.*, 1997; Cory and Hails, 1997). The importance of ecological testing and modelling is highlighted and its use for inferences on the fate of novel baculovirus genotypes (is the genotype prone to extinction in time? or will it be established as a member of natural populations?) and on the fate of hosts with varying sensitivities is briefly considered. Molecular biology techniques not only offer new insights into the mechanisms of virus host interactions, they also provide methods of its modification with some impact on safety perception. The potential of strain detection and identification also provides for novel details of monitoring in space and time suitable for direct comparisons of different genotypes, differing in subtle biological parameters.

The generation of a novel quality of (predictive) knowledge about baculovirus fate and effects seems to represent a significant scientific challenge in baculovirus research. The potentials and inherent limits of an intermediate stage of microcosm research still have to be explored in response to such a challenge. There is no doubt that strategies of baculovirus use as insecticides will profit from improvements in ecological knowledge in the long run.

## B. Experimentation and uses in greenhouses

Experimentation in closed environments is not generally used to improve control over experimental parameters, to confine experimentation from the open environment, or to generate predictive knowledge about the environmental behaviour of viruses. The development of a virus application in greenhouses is cited here to illustrate the stepwise extension of experience and knowledge during the conventional steps for the commercialisation as a biocide product. In this case, greenhouses merely represent the intended localisation for the application and do not constitute "simulated environments". The design of experimentation thus is not adapted to generate novel potentials of inferences about the fate and behaviour of the virus in the open environment.

The intended use in greenhouse cultures triggered experiments studying the efficacy of a nucleopolyhedrovirus, SeMNPV, for controlling larval populations of the noctuid *Spodoptera exigua*. Application of  $1 \times 10^8$  OBs per m<sup>2</sup> on ornamental plants and tomatoes resulted in 95% - 100% larval mortality. Mortality of early and late instar larvae was similar, but feeding damage was more pronounced when larvae were in late instars at the time of application. This is referred to as "maturation resistance". It was concluded that the virus was an effective potential control agent for *S. exigua* in greenhouses also in comparison with a use of chemical insecticides (Smits *et al.*, 1987b). Superior activity of baculoviruses against pests of ornamental plants in comparison to chemical treatments has also been reported by others (Geissler and Schiephake, 1991).

Different virus strains, known or suspected to be infective to specific target insects, have been screened with respect to their virulence (lethal dose, LD<sub>50</sub>). Selection of a strain for application may then include other criteria like the knowledge about host range and the possibility of cost effective production (Smits and Vlak, 1988a). Monitoring included the observation of movement and feeding

behaviour of the target organism to adapt application techniques (Smits *et al.*, 1987a). After optimisation of production conditions, the product was characterised for its biological activity and its microbiological composition and purity (Smits and Vlak, 1988b). After the accumulated information was considered to be satisfactory by all parties, registration of the product was the outcome (Smits and Vlak, 1994). Data of virus dispersal, virulence, and replication together with features of the host organism (development, susceptibility of instar stages, movement) provided input for modelling. Understanding viral population dynamics in generating predictive knowledge was considered to be useful for safety assessment in general and to contribute to the optimisation of application strategies (Moed *et al.*, 1990; van der Werf *et al.* 1991).

## **6. History of Use (Examples of Environmental Applications of the Organisms and Information Derived from These Examples)**

### **A. Brief historical account of baculovirus detection and application**

Observations of silkworm (*Bombyx mori*) cultures with descriptions of jaundiced larvae due to paralysis, as described in ancient Chinese literature, appear to be the earliest accounts describing the effect of baculovirus infections. In western literature, this is credited to the verses of the poem “De Bombyce” by the Italian bishop Marco Vida of Cremona, 1527 (Benz, 1986). Liquefaction of diseased larvae occurs in the process of converting insect biomass into progeny virus and the older literature refers to these symptoms as “melting” or “wilting”.

The breakdown of the European silk industry and virus infections threatening the emerging shrimp aquaculture industry provide two of the very few examples of negative consequences from baculovirus infectivity as perceived by mankind (Chen *et al.*, 1989; Chou *et al.*, 1995; Momoyama and Sano, 1996). Favourable conditions for epizootics in artificial mass cultures of arthropods contribute to this type of observation. Considering present knowledge of the widespread occurrence of baculoviruses in insects, their persistence and spread by a variety of vectors which exposes soil, crop plants and plant debris with great numbers of virus particles (Heimpel *et al.*, 1973), all day life can be regarded as natural “nontarget-species testing”. This occurrence demonstrates to some extent the lack of infectivity and pathogenicity of baculoviruses for organisms other than arthropods, in particular vertebrates including humans.

The first attempts to use baculoviruses for biological control can be dated back to the year 1892. During massive population increases of nun moths (*Lymantria monocha*, L.), a severe pine pest in Europe, the use of the infectious agent causing the so called “Wipfelkrankheit” was intended to combat the insect pest. But the “insecticide” was prepared in a manner corresponding to its identification as a bacterium at that time. Thus, the observation of diseased larvae and a final collapse of the population short after application must be attributed to a common natural outbreak of an epizootic, not to a successful application strategy (Huber, 1986).

The history of integrating novel experimental techniques and increasing knowledge in biology to finally identify the nature and features of the viruses did not proceed in a continuous and straightforward manner (Benz, 1986). The systematic exploration of the potential of baculoviruses for the control of insect pests is described by Benz (1986) and Huber (1986). Yearian and Young (1982) and Cunningham (1982) documented the use of about 50 different baculoviruses. The successful biological control of insect pests in field crops, plantation, orchard crops, and forests was demonstrated by field experimentation and applications of widely different scales all over the world. These experimentations and applications resulted in the development of fully registered baculovirus insecticides in several countries. However, registration and commercialisation of viral pesticides did

not keep pace with the development, although, it was estimated that about 30% of the major insect pest species could potentially be controlled by baculoviruses (Falcon, 1978).

A substantial increase in the use world-wide did not fundamentally change the situation regarding the registration and commercial production of baculovirus insecticides and their economic success. Although environmental policy and public awareness demand an increasingly rigorous testing for the registration of chemicals, a trend that should generally favour biologicals, the situation regarding the registration and commercial production of baculovirus insecticides has not changed fundamentally. Baculoviruses, due to their character as biologicals, are endowed with a variety of application constraints. Among these, the narrow host range, a limited life time and the slow speed of action resulting in demands on application strategies, are important factors in their failure to effectively compete with chemicals (Huber, 1986; Bohmfalk, 1986). Two noticeable developments might, in the future, contribute to a more favourable situation for baculovirus insecticides on the market.

- The progress in the development of techniques for virus production may significantly improve their economic competitiveness. Supported by the development of cell culturing techniques and a widespread use of baculovirus systems for the expression and production of a multitude of heterologous proteins, a cost effective multiplication of viruses by insect cell cultures in fermenters has become a realistic perspective (Rhodes, 1996).
- Principles and methods of molecular biology contribute to an enhanced understanding of the interaction of baculoviruses with their hosts and offer a variety of potentials to modify this interaction with the objective to relieve some of the (biological) constraints on their convenient applicability (Bonning *et al.*, 1992; Crook and Winstanley, 1995).

The first trend can clearly be seen in an increasing number of publications on technical aspects of baculovirus insecticide production. Limitations on maintaining infectivity following growth in insect cell culture are becoming better understood and overcome. The exclusive emphasis given to the registration of genetically modified baculoviruses in a recent contribution on commercialisation of baculovirus insecticides, appears to underpin the importance given to the latter trend internationally (Black *et al.*, 1997).

## B. Types of pest control and strategies of virus selection for application

Any intended use of baculoviruses for insect pest management includes the screening for a virus strain virulent for the particular species. Isolates from diseased insects in the application area frequently are the first choice and are used as a promising and convenient starting point. If available, such isolates are in general included in the screening, but the testing for suitable viruses is not conventionally limited to those indigenous agents (Smits and Vlak, 1988a; Shapiro and Robertson, 1991). Both, Benz and Huber (1986) give a special credit to the control of the European spruce sawfly, *Gilpinia hercyniae*, introduced in the US and Canada long before the 1930s, when it became a serious pest of spruce. Population gradations frequently collapsed after the dissemination of a NPV which was probably accidentally introduced with imported parasites. Later, in the following, planned applications successfully supported the natural epizootics and the establishment of the virus finally controlled areas of minor infestations. The CpGV-isolate which is registered and commonly used for the control of the codling moth in different European countries, was originally isolated from diseased insects found in Mexico (Tanada, 1964).

This instance can be taken as an example of two types of strategies of biological control: the **introduction and establishment of a novel agent from a different geographic region**, however not planned in this case, and **inoculative augmentation**, used to enhance a natural antagonist, in order to

achieve control of the pest species. The history of baculovirus introductions or uses does not contain any documented observations of negative or unintended consequences. This is in contrast to other introductions for biological control (Ehler, 1991) and reflects the biological properties of these viruses to some extent.

The application of baculoviruses in forests can most often be characterised as an **augmentation** of an indigenous pathogen reservoir to improve natural control over high rates of insect multiplication (radiations). This type of application represents the most impressive record of the use of baculoviruses with respect to extent and success (Huber, 1986). The control of insect pests in orchards, greenhouses and annual crops, in general, demands a third type of application, **inundative augmentation**, by inoculating these areas with an amount of viruses that productively infects the number of pest organisms that must be controlled to keep the damage below economic threshold. Such applications do not rely on virus multiplication and spread to achieve the desired effect - and thus are more comparable to the use of topically applied chemicals. However, the slope of the rate effect curve and economic reasons will not allow management of the efficacy of applications by modifying (increasing) the application dose in the same way as is sometimes the case with chemicals (Huber, 1986).

The screening for virus isolates suitable for a pest management objective is generally not adapted to the intended type of control as categorised above. Infectivity and virulence, encompassing the dose-response function of the virus interaction with the pest species (C.23) make up the most important and frequently exclusive criteria for the selection of a virus and the planning of field application. Whereas bioassays serve in the analysis of the potency of a baculovirus preparation, field testing is essential to evaluate whether its use is feasible and to investigate the effect of formulations and the application conditions of the insecticide. World-wide, many improvements and modifications in production, formulation and application of baculovirus insecticides have been achieved and tested in recent years.

Before the era of molecular biology, the potential to modify infectivity and persistence of baculoviruses was frequently tested by conventional means of selection, eventually including mutagenesis (e.g. Reichelderfer and Benton, 1973). Eventually, selection resulted in a virus mutant analogous to genetically modified strains carrying a deletion of a gene for host interaction with the same objective. The description of a mutant, selected by Wood and co-workers (1981), which demonstrated a significantly reduced mean lethal time, suggests such a coherence (with genetically modified viruses lacking the *egt*-gene, see A 4.4 ). From experiments intending to modify host range or to adapt baculoviruses to alternate hosts, no clear cut picture has yet emerged. Experimentation before the introduction of molecular methods suffered from the difficulty of differentiating inoculum viruses from viruses persistent in the insect culture and potentially activated during the experiment. An adaptation to novel hosts has been described in several cases and/or an enhanced virulence was observed after virus multiplication in an alternative host (Shapiro *et al.*, 1982; Martignoni and Iwai, 1986a; Stairs, 1990).

The testing of virus multiplication in alternative hosts may also be triggered by the objective to improve virus production with respect to cost effectiveness. For example, salt marsh caterpillars (*Estigmene acrea*) appeared to be a suitable production host for several NPVs of forest pests (*Orygia pseudotsugata* SNPV, *Choristoneura fumiferana* MNPV) by not only improving virus yield per larvae, but also by enhancing virulence (Shapiro *et al.*, 1982). From experiments analysing the virulence of viruses obtained from different instar stages of larvae, it was recommended the virulence in and yield of occlusion bodies (OB) per weight of larvae for the optimisation of production yields be observed (Shapiro *et al.*, 1986). Considering the potential of virus evolution during multiplication in an unnatural environment with novel selective constraints, an observation of the biological activity of production lots obviously is advisable, especially after propagation in alternative hosts (Reiser *et al.*,

1993; Maracaja *et al.*, 1994). The optimisation of production in cell culture may represent an additional challenge in this respect (Lynn *et al.*, 1993; Tompkins *et al.*, 1988).

Apart from the modification or selection for host range or infectivity parameters, another biological feature of baculoviruses compromising their applicability in the environment seems to be prone to direct selection: that is their sensitivity for UV-radiation, being the most significant factor for the limited life time of baculovirus insecticides. Although the mechanism of improved UV adaptation is not elucidated, some reports of successful selections were published (*e.g.* Brassel and Benz, 1979; Witt and Hink, 1979; Shapiro and Bell, 1984). However, the use of such variants in field experimentation was not reported. Selective procedures were also used to isolate variants of *Spodoptera frugiperda* MNPV with an enhanced rate of vertical transmission of baculovirus infections from adults to progeny, for example, by egg contamination (Fuxa and Richter, 1991). Such variants are likely to modify the dynamics of the spread of infections in natural populations and might improve the control of pests in particular situations.

Baculoviruses have also been used in combinations of different virus strains, in order to achieve control of different pest species (Harper, 1986; Dhandapini *et al.*, 1992). The synergistic effect known from certain double infections (Arne and Nordin, 1995; Ding *et al.*, 1995, see section A.4.2) has not been exploited in field experimentation.

### C. Modifications of chemical composition of formulations and of application techniques

In addition to approaches intending to improve the use of baculoviruses by modifying some of their biological parameters, formulations were adapted to achieve an economically feasible control. In competition with chemical insecticides, field testing and applications world-wide are evaluated for the optimisation of pest control.

The limited lifetime of baculovirus insecticides in the environment, to a great extent caused by their sensitivity for sunlight (UV), can be increased by the addition of a variety of UV-protectants. Optical brighteners, originally only tested as radioprotectants also proved to very significantly enhance virulence of virus host interactions in the laboratory (Shapiro, 1992; Shapiro and Robertson, 1992). The median lethal dose was reduced up to 4 orders of magnitude, the median lethal time was reduced for about 50% (7 instead of 14 days), and non-susceptibility was converted to susceptibility in some cases (Shapiro and Dougherty, 1994; Zou and Young, 1996). Laboratory testing was also supplemented by field testing with qualitatively corresponding results (Webb *et al.*, 1994; Zou and Young, 1996). The phenomenon obviously requires the combined action of brighteners and viruses. At the highest dosage, the application of brighteners alone does not induce larval death. In field tests the increase of mortality of *Lymantria dispar* caused by the indigenous natural virus was observed (Webb *et al.*, 1994).

In order to enhance the frequency and effectiveness of exposition of insect larvae, which predominantly become infected by feeding on plant material, spraying techniques have to be adapted and optimised (Smits *et al.*, 1988; Payne *et al.*, 1996). The addition of detergents and stickers serves for modifications of the distribution of spray droplets, and feeding attractants (*e.g.* crude sugar) are tested in order to enhance productive encounters of larvae with this distribution. The requirement to carefully test the environmental safety of formulation additives is highlighted by a field observation that molasses added as an UV protectant proved to be phytotoxic for soybean plants (Im *et al.*, 1990).

A variety of approaches describe the combined use of baculoviruses and chemical insecticides at significantly reduced application dosages (1/3 - 1/10). Apparently, the chemicals reduce the tolerance of insects for other stress factors, thereby enhancing their susceptibility for baculovirus infection.

Alternatively, an impact of the virus infection on the insects ability to degrade the chemical has also been suggested as a mechanism (Huang and Dai, 1991). A synergistic (more than additive) enhancement in effectiveness of a combined use has been described (Jacques *et al.*, 1988, 1989; Salama and Moawed, 1988; Peters and Coaker, 1993). This strategy was particularly recommended to compensate unsatisfactory control due to heavy insect infestations or climatic conditions (Moscardi and Corso, 1988; Ding *et al.*, 1989).

#### **D. Field testing and commercial use of baculovirus insecticides**

A detailed continent-by-continent survey on the developmental, experimental and commercial use of baculovirus insecticides was recently compiled in "Insect Viruses and Pest Management" (Hunter-Fujita *et al.*, 1998). Some examples of the most important baculovirus insecticides tested and used in the field are:

- *Adoxophyes orana* GV has been extensively field tested and finally registered in Switzerland for control of the summerfruit tortrix (Andermatt, 1991). This virus is also registered in Germany.
- *Agrotis segetum* GV and NPV have been tested in many countries for control of the common cutworm (*A. segetum*) and the greasy cutworm (*A. ipsilon*). They showed superior efficacy compared to chemical insecticides. *Agrotis segetum* GV-based bioinsecticides were registered in Denmark and the former Soviet Union (Huber, 1998; Lipa, 1998).
- *Anticarsia gemmatilis* MNPV has been used for control of the velvetbean caterpillar in soybean on a large scale in Brazil (Moscardi, 1990; Da Silva, 1992). The application of AgMNPV has increased from 2000 ha treated in 1982/ 1983 to 1 million ha in 1989/90. At a dose of 50 LE/ha (LE=Larval equivalents, the amount of virus prepared from one larvae) one application of the baculovirus was as efficient as chemical control (frequently requiring several applications) to maintain population densities of *Anticarsia gemmatilis* below economic threshold. The combination of the virus preparation with low dosages of chemical insecticides has also been tested and proved to enhance effectivity. It was recommended to be used in situations of heavy infestations when control cannot be achieved with the virus preparation alone (Moscardi and Corso, 1988; Da Silva, 1995). The widespread use of the viral insecticide was the reason to initiate the special monitoring of Brazilian *Anticarsia* populations for the selection of phenotypes with enhanced resistance to the virus (see C.24).
- *Cydia pomonella* GV has been extensively tested for control of the codling moth in apples, pears and walnut in the last 25 years. Meanwhile CpGV based products are registered in many European countries, e.g. Austria, France, Germany, Spain, Switzerland and the Netherlands and have been registered in the US since 1995. Field testing has also been conducted in Australia, Canada, Chile, the USA and other countries. Best results were obtained in cooler climates where the codling moth produces only one annual generation (Audemard *et al.*, 1992; Huber, 1998).
- *Autographa californica* MNPV has a broad host range and has been tested for control of different pest insects, esp. of *H. virescens* and *T. ni*. AcMNPV has been registered in the USA since 1994. Experimental and commercial use of AcMNPV is reported from Central America, where it has been applied on several thousand hectares of cabbage and broccoli (Hunter-Fujita *et al.*, 1998). In recent years, field tests have been performed using genetically engineered AcMNPV recombinants with improved speed of kill. These recombinants, which

express insecticidal toxins and/or lack the *egt* gene, are the prototypes for other genetically engineered baculovirus insecticides (Cory *et al.*, 1994, Black, 1997).

- *Heliothis (Helicoverpa) sp.* NPV. The cotton bollworm (*Helicoverpa zea*) and the tobacco budworm (*Heliothis virescens*) are major pests of cotton in southern/south-eastern regions of the US and serious pests of many food, fibre, and forage crops world-wide. *Heliothis* SNPV based insecticides were developed during the late 1960s - 70s, registered in 1975, and were a commercial success until the early 1980s when synthetic pyrethroids were introduced. The total area in the USA sprayed with this virus was estimated to be more than 1 million hectares (C. M. Ignoffo cited in Cunningham, 1998). A novel strategy for control of *Helicoverpa ssp.* using *Heliothis* SNPV was investigated when it was found that introduced and native early season host plants (in particular the wild geranium, *Geranium dissectum*), occupying only 5% of the Mississippi rural area, support the first generation of bollworm/budworm populations which subsequently invade the cotton fields. A control of the first and possibly second generation by applying HzSNPV to alternate hosts had been tested in small field tests as an effective potential management strategy. Large area testing followed these first experiments (Bell and Hardee, 1994; Bell and Hayes, 1994; Hayes and Bell, 1994).
- *Helicoverpa armigera* NPV has a great potential for the control of African cotton bollworm, *H. armigera*, which is one of the most deleterious insect pest in warmer climates of the Old World. Extensive studies including combinations with another biological, *Bacillus thuringiensis* (Bt), have been performed in China, India and African countries (Zhang *et al.*, 1996a; 1996b, Kunjeku *et al.*, 1998). A significantly reduced mean lethal time and a high efficacy (97 % of larval deaths) were observed for the combination with Bt, which also had an effect on pupal mortality in the following year. The effectiveness was found to be at least equivalent to that of recommended chemical insecticides.
- *Lymantria dispar* NPV has been widely applied for control of gypsy moth *L. dispar* in the USA, where it is registered since 1978 and where more than 11 000 ha have been treated (for review see Lewis, 1981; Podgwaite, 1985; Cunningham, 1998). It also became the most important viral insecticide in the former Soviet Union (for review see Lipa, 1998). In order to improve the control of divergent densities of the gypsy moth, the commercial product Gypchek was modified with sunlight protectants, feeding stimulants and a sticker. Two applications of  $1.25 \times 10^{12}$  OB/ha resulted in a reduction of egg masses by 98% and 80% in comparison with control woodlots at different sites (Podgwaite *et al.*, 1992). This application dose is currently recommended for the use of LdMNPV preparations in the USA.
- *Mamestra brassicae* NPV has been tested for efficacy in a number field trials for control of the cabbage looper, *M. brassicae*. This virus has a considerably broad host range and more than 30 susceptible insect species have been identified (Doyle *et al.*, 1990). Reasonable success was reported for the control of *P. xylostella*, *Heliothis spp.*, *Spodoptera spp.*, *Trichoplusia ni* and others. Commercial products were developed and registered in France and the former Soviet Union (Hunter-Fujita *et al.*, 1998).
- *Neodiprion sertifer* NPV has been successfully applied for control of the European pine sawfly, which is a serious pest on pine plantations (for review see Cunningham and Entwistle, 1981). Field work with NeseNPV has been conducted in Canada, Scandinavia, Poland, UK, the former Soviet Union and USA. Commercial products were registered in Finland (1983), UK (1985), USA (1983, but discontinued by the company in 1991) and the former Soviet Union.

- *psuedotsugata* NPV is used and has been registered since 1976 in the US and Canada for control of the Douglas fir tussock moth. It is recommended particularly for early control when population densities are relatively low because it takes five to eight weeks before the larvae stop feeding, during which time further defoliation occurs. (Defoliator Management Guidebook, Ministry of Forests, British Columbia, Canada <http://www.for.gov.bc.ca/tasb/legsregs/fpc/fpcguide/guidetoc.htm>).
- *Spodoptera* spp. NPV. Several specific viruses have been isolated from different *Spodoptera* species, such as SeMNPV from the beet army worm (*S. exigua*), SpexNPV from the African armyworm (*S. exempta*), SfMNPV from the fall army worm (*S. frugiperda*), SpltNPV from the Tobacco cut worm (*S. litura*), SpliNPV from the Egyptian cotton leaf worm (*S. littoralis*) and others. Extensive research on these viruses and field tests resulted in the development of several commercial products (Hunter-Fujita *et al.*, 1998). Field isolates of SeMNPV were found in the USA, Thailand and Spain (Caballero *et al.*, 1992). A registered product, based on the US isolate, is registered and widely used in Dutch greenhouses and in the USA, where *S. exigua* was found to be tolerant to many chemical insecticides. SfMNPV is being used in Central and South America, where *S. frugiperda* occurs as a major pest of corn and rice. It was reported that more than 20 000 ha were treated with SfMNPV in Brazil by 1992 (Oliveira *et al.*, 1998).

## 7. Characterisation of the Genomes (e.g. Open Reading Frames, Insertion Sequences), and Stability of These Characteristics

### A. The structure of the baculovirus genome

The baculovirus genome is a double-stranded, circular DNA which varies between 90 - 160 kilo base pairs (kbp) for different members of the family Baculoviridae (Murphy *et al.*, 1995). Based on the analysis of the entire genome sequence of AcMNPV, it was estimated that the genome contain between 140 - 160 genes. About half of these genes have been transcriptionally and functionally characterised (for review see Kool and Vlak, 1993, Possee and Rohrmann, 1997). The immense variation of genome size among different baculovirus species suggests that some baculovirus genomes contain significantly less genes than others. Apart of these interspecific differences of genome size, an intraspecific variation caused by natural variation (Chapter 7.2) and host transposon insertion (Chapter 7.3) can be observed. The ability of the baculovirus nucleocapsid to expand and to harbour additional genetic information was exploited and led to the development of the baculovirus expression vector system.

To date, the genomes of three baculoviruses isolated from noctuid hosts have been completely sequenced. These are AcMNPV (with a genome of 133,894 bp), BmNPV (128,413 bp) and OpMNPV (131,990 bp) (Ayres *et al.*, 1994, Maeda, 1994; Ahrens *et al.*, 1997). Other complete baculovirus genome sequences can be expected in the future. Partial sequence information of about 35 other baculovirus genomes is available on the GenBank database (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>).

In general, baculovirus genomes contain non-overlapping open reading frames (ORFs) with short intergenic regions. However, a few ORFs which partially overlapped adjacent ORFs were found. For example, in the genome of several NPV and that of *Cryptophlebia leucorreta* GV, the 5' end of the adjacent, bidirectionally orientated ORFs encoding *p38* and *lef-5* overlap to a considerable extent (Ayres *et al.*, 1994, Jehle and Backhaus, 1994). Gene splicing has only been observed with the early activator *ie-0* of AcMNPV. Its coding region consists of a small exon and the ORF of *ie-1*, which are spliced together and expressed early in infection (Kovacs *et al.*, 1991).

Interspersed homologous regions, known as hr regions, have been identified in many baculovirus genomes. The hr regions in the AcMNPV genome consist of multiple copies of 28-30-bp palindromic sequences flanked on either side by direct repeats of 20 bp (Cochran and Faulkner, 1983; Ayres *et al.*, 1994). These regions function as cis-acting enhancing factors for the transcription of early genes by RNA polymerase II (Theilmann and Stewart, 1992, for review see Friesen 1997) and as origins of DNA replication in transient DNA replication assays (Pearson *et al.*, 1992; for review see Kool *et al.*, 1995).

## B. Natural variability of baculovirus genomes

In the 1970s, progress of the analysis of baculovirus genomes was accelerated: (i) the development of *in vitro* and *in vivo* cloning procedures, which allowed researchers to isolate and to propagate single virus genotypes, and (ii) the introduction of restriction endonuclease analysis, which allowed isolated virus DNA to be cleaved into fragments of specific length.

The occurrence of submolar bands in the restriction enzyme profiles of isolated DNA of baculovirus field isolates provided first evidence of substantial genetic variation within the natural baculovirus population. When single genotypes were isolated using plaque purification assays, these differences were commonly mapped to point mutations, acquisition or deletion of restriction sites or to insertions or deletions of hundreds or thousands basepairs (Lee and Miller, 1978; Crook *et al.*, 1985; Smith and Crook, 1993).

## C. Transposon insertion into baculovirus genomes

Horizontal transfer of insect host transposable elements (TE) into baculovirus genomes is an occasionally observed event that contributes to genetic heterogeneity. Most TE insertion of AcMNPV or GmMNPV were associated with a specific alteration of plaque morphology of infected insect cells and characterised by a significantly reduced number of polyhedra per infected cell (Fraser and Hink, 1982; Fraser, 1986). These so-called “few polyhedra” or FP mutants were generated during serial passage and harboured transposon insertion within the 25K gene commonly known as FP locus (map unit 36.0 - 37.0). The role of the 25K gene has not yet been completely elucidated but it might be involved in the temporal regulation of BV and ODV production (Jarvis *et al.*, 1992). AcMNPV with a mutated 25K gene was frequently impaired in virion occlusion and intranuclear nucleocapsid envelopment but released up to five-times more BV from infected SF-9 cells (Harrison and Summers, 1995). Disruption of the 25K gene apparently favours the production of budded viruses (BV). Since BV are about three orders of magnitude more infectious to cultured cell lines than ODVs, it is likely that transposon carrying FP-mutants have a strong selection advantage over wild-type viruses during the specific conditions of serial passaging in cultured cells.

A number of FP mutants of AcMNPV and GmMNPV harbouring transposons which belong to the class of (DNA)-transposons have been described (Table 4.3). These transposons originate from *T. ni* or *S. frugiperda* host cells. Although their sizes vary from 0.3 - 2.5 kbp, they share common structural characteristics as short terminal inverted repeats of 13-15 bp and a duplication of a TTAA target sequence (for review see Fraser, 1986; Friesen, 1993; Jehle, 1996). Most of these transposons lack open reading frames to encode a transposase suggesting that they are defective copies that cannot transpose autonomously. Only transposon piggyBac (formerly termed IFP2) was shown to contain an ORF which supports insertion and excision of this element (Fraser *et al.*, 1995; Elick *et al.*, 1996).

The conformation that transposon insertion into baculovirus genomes is not restricted to the specific conditions of cell culture but also occurs during normal infection of host larvae was provided

by Jehle *et al.* (1995) who demonstrated the insertion of two different Tc1/mariner-like transposons into CpGV genome after infection of *C. pomonella* and *C. leucotreta* larvae.

Host transposon insertion into baculovirus genomes exerts drastic effects on the genomic integrity of the virus and has possibly far reaching consequences for baculovirus evolution. The observed effects are (i) acquisition of new functional genes and/or regulatory sequences, (ii) disruption of viral genes rendering them non functional, and (iii) deletion of extended portions of the genome by self-recombination or excision (Carstens, 1987). In most cases, transposon insertion can result in reduced fitness and competitiveness of the altered virus. However, the isolation of the FP-mutants and the CpGV mutants containing a transposon have demonstrated that these viruses are viable and that transposon insertion significantly increases genetic heterogeneity.

**Table 4.3 Insect host transposons found in baculovirus**

Transposon	Host Virus	Insect Origin	Reference
TED (retrotransposon)	AcMNPV	<i>T. ni</i>	Miller and Miller, 1982; Friesen and Nissen, 1990
M5	AcMNPV	<i>S. frugiperda</i>	Carstens, 1987
IFP2 (piggyBac)	GmMNPV	<i>T. ni</i>	Cary <i>et al.</i> , 1989
TFP3 (tagalong)	AcMNPV	<i>T. ni</i>	Wang <i>et al.</i> , 1989
TFP3 (tagalong)	GmMNPV	<i>T. ni</i>	Wang <i>et al.</i> , 1989
IFP2.2	AcMNPV	<i>S. frugiperda</i>	Beames and Summers, 1990
IFP1.6	AcMNPV	<i>S. frugiperda</i>	Beames and Summers, 1990
E	AcMNPV	<i>S. frugiperda</i>	Schetter <i>et al.</i> , 1990
hitchhiker	AcMNPV	<i>T. ni</i>	Bauser <i>et al.</i> , 1996
TCI4.7	CpGV	<i>C. leucotreta</i>	Jehle <i>et al.</i> , 1995
TCp.3.2	CpGV	<i>C. pomonella</i>	Jehle, 1996

## 8. Genetic Transfer Capability

### A. Recombination

Recombination is a decisive mechanism which is responsible for intergenomic exchange of information in all organisms, including baculoviruses. There are two different classes of recombination, a homologous and a non-homologous or illegitimate recombination. Homologous recombination means the genetic exchange of allelic sequences between two different genomes, whereas non-homologous recombination comprises all events of non-allelic sequences exchange.

#### *Homologous recombination*

With baculoviruses, extensive homologous recombination has been observed between the closely related AcMNPV, GmMNPV, RoMNPV and TnMNPV (Miller *et al.*, 1980; Smith and Summers,

1980; Croizier and Quiot, 1981). After coinfection of TN-368 cells of *T. ni* with AcMNPV and RoMNPV seven out of 100 isolated plaques were identified as recombinants, each with at least one crossing over event (Summers *et al.*, 1980). Intraspecific recombination was also observed when SF-9 cells were co-infected with different pairs of AgMNPV genotypes (Croizier and Ribeiro, 1992). These results indicated that the frequency of recombination among highly homologous baculoviruses is considerable.

When Croizier *et al.* (1988) co-transfected larvae of *Galleria mellonella* with isolated DNA of RoMNPV and restriction fragments of AcMNPV, a very high number of RoMNPV recombinants but no parental RoMNPV genotypes were recovered. Since replication of AcMNPV in *G. mellonella* is much more effective than that of RoMNPV, it was suggested that the RoMNPV might have acquired an unknown replication advantage by recombination with AcMNPV DNA fragments.

A high recombination rate was also found when *T. ni* larvae were co-infected with wild-type AcMNPV and a polyhedrin-negative, lacZ-expressing AcMNPV mutant. A recombination frequency of at least 6.6% was estimated by plaque assays of hemolymph of co-infected larvae and scoring for recombinant plaques (Merryweather *et al.*, 1994).

Further evidence for extensive homologous recombination among different variants of the same virus species is based on the isolation and restriction mapping of wild-type virus mixtures. By the method of *in vivo* cloning Smith and Crook (1988; 1993) isolated a number of different ArGV genotypes which differed in a few restriction fragments. Based on comparative physical mapping, it was suggested that the diversity of these variants was partly caused by recombination during natural coinfections. A rapid replacement of two closely related parental SeMNPV variants (Se-US and Se-SP2) by a recombinant (Se-SUR1) which differed from the parental strains by several cross over events was observed after coinfection of *S. exigua* larvae. This result suggested selection advantage of the new recombinant over the parental strains, though significant differences in the biological activities (LD<sub>50</sub> and LT<sub>50</sub>) were not observed (Munoz *et al.*, 1997).

These findings underscore the fact that homologous recombination among virus variants is a normal process of genetic exchange which occurs at a considerable frequency during *in vitro* and *in vivo* replication. The process of homologous recombination appears to be linked to and driven by the viral replicative machinery. Although the genetic factors involved in recombination have not been identified, it was suggested that genes involved in DNA replication and possibly hr regions may play a central role in the events (Martin and Weber, 1997).

### ***Heterologous recombination***

The probability of recombination between baculovirus sequences decreases drastically when the baculoviruses are less closely related. So far, recombination between different baculovirus species has only been observed in laboratory experiments where the generation of recombinants was biased by selection or replication advantages. For example, the identification of genes encoding host range factors or apoptosis inhibitor was achieved using this approach (see A 3.3 and A 4.3).

Heterologous recombination has been forced by co-transfection of restricted DNA or cloned DNA fragments with intact viruses or virus DNA. In an experiment addressing the possibility of interspecific recombination Roosien *et al.* (1986) co-transfected *S. frugiperda* cells with a polyhedrin negative AcMNPV mutant and a plasmid containing the polyhedrin gene of MbMNPV. AcMNPV mutants were isolated and contained the MbMNPV polyhedrin gene insertion at different, non-specific sites. However, these recombinants contained less virions and showed reduced infectivity to *S. frugiperda* cells than wild-type AcMNPV. Similar results were obtained when a polyhedrin minus

AcMNPV mutant was co-transfected with a cloned polyhedrin gene of SfMNPV (Gonzales *et al.*, 1989). The recombinant isolated from this experiment expressed less than 25% of the level of wild-type AcMNPV polyhedrin gene.

In general, the competitiveness and biological fitness of heterologous recombinants is lower than that of wild-type viruses. One reason is that by non-specific recombination events, coding or regulatory sequence functions might become impaired resulting in a less viable virus.

## II. Human Health Considerations

Baculoviruses are naturally occurring pathogens of arthropods. Their host range is exclusively restricted to arthropods. No member of this virus family is infective to plants or vertebrates. Baculoviruses are ubiquitously present in the environment and have been used for biological insect control for more than 100 years. Circumstantial evidence for the safety of baculoviruses emerges from the history of contact between baculoviruses and humans without any detrimental effect.

During the 1970s, the US Environmental Protection Agency established the Guidance for Safety Testing of Baculoviruses, which also became a guideline of baculovirus safety tests in many other countries (Anonymous, 1975; Summers *et al.*, 1975). This guidance included *in vivo* and *in vitro* safety studies and was applied for commercial baculovirus insecticides, such as *Helicoverpa zea* SNPV (Elcar®), *Orgyia pseudotsugata* NPV (TM Biocontrol-1) and many others. *Helicoverpa zea* SNPV was the first commercial baculovirus insecticide and is one of the most extensively tested entomopathogenic viruses (Ignoffo, 1975). Safety tests of more than 51 entomopathogenic viruses including more than 30 baculoviruses resulted in a long and complete safety record (extensively reviewed by Ignoffo, 1973; Burges *et al.* 1980a, 1980b; Gröner, 1986). No adverse effect on human health has been observed in any of these investigations indicating that the use of baculovirus is safe and does not cause any health hazards.

### A. Safety tests of baculoviruses included

#### *In vitro and in vivo replication of baculoviruses in vertebrate and mammals*

The possibility of replication of baculoviruses in vertebrates and mammals was investigated by challenging many vertebrate and human cell lines with OB and BV of many baculoviruses. Although virus uptake of these cells was frequently reported, no evidences of virus replication or cytopathological effects were observed. The few early reports, which stated baculovirus replication in vertebrate cell lines (Himeno *et al.*, 1967; McIntosh and Shamy, 1980) could never be demonstrated or confirmed in other laboratories. After oral uptake of baculoviruses by man, mice, chickens, rabbit, pigs, and other mammals, no specific antibody production, which would indicate replication of the virus used to challenge the host, was observed (reviewed by Gröner, 1986). In contrast, a specific immunological response against CpGV was observed in woodmice (*Apodemus sylvaticus*) which were trapped in an apple orchard sprayed with CpGV. It is conceivable that an antigenic challenge may have occurred via the nasal mucous membrane, virus replication or a negative effect to the animals was not observed (Bailey and Hunter Fujita, 1987).

Using a recombinant AcMNPV containing the *cat* gene under the control of the Rous sarcoma virus terminal repeat promoter and the  $\beta$ -galactosidase gene under the control of the very late polyhedrin promoter reporter gene, expression was analysed in different invertebrate and vertebrate cell lines (Carbonell *et al.*, 1985; Carbonell and Miller, 1987). No *cat* or  $\beta$ -galactosidase activity was detected in transfected mouse or human carcinoma cells. On the other hand, recent reports showed that recombinant AcMNPV virus is efficiently taken up by human hepatocytes via an endosomal pathway.

Recombinant AcMNPV carrying the *Escherichia coli lacZ* reporter gene under control of the Rous sarcoma virus promoter and mammalian RNA processing signals showed considerable expression levels in the human liver cell line HepG2, but at very low levels, or not at all, in cell lines from other tissues (Hofmann *et al.*, 1995; Boyce and Bucher, 1996). Based on these findings it was suggested that baculovirus might be exploited for liver-directed gene therapy. From the view of baculovirus safety this results also show that careful attention has to be paid to the promoters used to control heterologous gene expression in recombinant baculoviruses.

#### ***Acute oral and intraperitoneal toxicity of mammal***

Acute toxicity of *Helicoverpa zea* NPV has been tested in many mammals, *e.g.* rat, mouse, rabbit, guinea pig and man, at doses from  $6 \times 10^9$  to  $3 \times 10^{12}$  OB/kg, which is up to 1000 times the average field rate per acre. Similar tests were conducted with TnSNPV, SeMNPV, AcMNPV, LdMNPV, CpGV and others (Ignoffo, 1975).

#### ***Subacute dietary-administration to mammals***

In order to test potential subacute toxicity or pathogenicity *Helicoverpa zea* NPV was fed or subcutaneously injected to mice (about  $5 \times 10^{10}$  OB/kg of animal), rats ( $4 \times 10^9$  to  $4 \times 10^{11}$  OB/kg), beagle dogs ( $7 \times 10^9$  OB/kg), rhesus monkeys ( $10^8$  to  $1.6 \times 10^{10}$  OB/kg) and man ( $10^9$  OB/day for 5 days). Similar tests were performed with rats for OpMNPV, LdMNPV and others (Ignoffo, 1975). Furthermore, health monitoring of workers who were involved in production of HzSNPV (for the viral pesticide) for extended time periods did not show any clinical symptomatology, nor any serological response or any indications that the virus is allergenic (Rogoff, 1975).

#### ***Eye- or skin-irritation to mammals***

Eye irritation tests were negative, when  $1 \times 10^5$  to  $20 \times 10^5$  OB/eye were applied to rabbit eyes. Skin irritation sensitivity tests were conducted with *Helicoverpa zea* NPV in rabbits, guinea pigs and man at doses of  $10^3$  to  $10^6$  OB/mm<sup>2</sup> of skin. Dermal and eye applications have been also conducted with *Neodiprion sertifer* NPV, AcMNPV, SeMNPV and others without any adverse reactions (Ignoffo, 1975).

#### ***Cytogenetical implications as chromosomal aberrations or sister chromatid exchange to mammalian cell lines***

In studies on the activation of endogenous C-type retrovirus by baculoviruses in three mammalian cell lines (mouse, rat, and man) no activation of C-type retrovirus could be detected (Schmidt, 1981). When cultured frog cells (ICT-2A) were challenged with TnSNPV no virus multiplication and no chromosome aberrations were observed over a 4-week period of time (McIntosh, 1975). No chromosome aberrations in Chinese hamster cells, mouse cells after oral uptake of BV or OB of AcMNPV and MbMNPV was observed (Miltenburger, 1978).

#### ***Carcinogenicity, teratogenicity, mutagenicity to mammals.***

Potential carcinogenicity of *Helicoverpa* NPV were conducted in mice  $10 \times 10^9$  to  $4 \times 10^{11}$  OB/kg or rats ( $3.5 \times 10^{12}$  OB/kg), teratogenicity tests were performed in rats at a dose of  $10^9$  OB/kg. No evidence of carcinogenic or teratogenic effects was found (Ignoffo, 1975).

### **III. Environmental and Agricultural Considerations**

#### **1. Natural Habitat and Geographic Distribution. Climatic Characteristics of Original Habitats**

Baculoviruses are ubiquitous in the environment, their prevalence depending on the frequency of occurrence of their arthropod hosts that inhabit terrestrial and marine ecosystems. By 1986, about 1100 viruses known to infect insects had been described, more than 60% of them being baculoviruses (Martignoni and Iwai, 1986b). Natural epizootics and their survival under a variety of environmental / transport conditions provides for particularly high densities in diverse locations in the environment which may act as infection sources for pathogen multiplication (cabbage leaf, dust: Heimpel *et al.*, 1973; Olofsson, 1988). Viruses from the same arthropod species from different geographic regions of the world may be as closely related as their hosts as demonstrated by the similarity of their restriction pattern (Vickers *et al.*, 1991). However, different baculoviruses can also be isolated from the same species in one geographical region. Restriction endonuclease digest patterns can be used to analyse the genetic heterogeneity of baculovirus strains and of strains obtained from different geographical regions (Laitinen *et al.*, 1996).

#### **2. Significant Involvement in Environmental Processes, Including Biogeochemical Cycles and Potential for Production of Toxic Metabolites**

The ecological significance of baculoviruses is characterised by their impact on the population dynamics of their arthropod hosts. As deduced from experiences with epizootics of insects they can be regarded as a regulator of their host population density and multiplication. No report on a potential contribution of baculoviruses to the extinction of insect species is available. This feature does not seem to depend only on the usual evolutionary adaptation of the interaction of pathogens with their hosts, but might be a more fundamental property of their life style. Through international trade, and in the course of applications of baculoviruses for pest control to a considerable extent, strains of baculoviruses have been moved around the globe without negative observations on local faunas. The ecological traits of baculoviruses (in particular virulence and dispersal characteristics) in their combination are an apparent biological constraint on excessive population increases, potentially endangering the basis of their existence, and the survival of their arthropod hosts.

With the impact on population densities of their hosts, a secondary effect on predators, parasites and hyperparasites is evidently connected.

#### **3. Pathogenicity – Host range, Infectivity, Toxigenicity, Virulence, Vectors**

##### **A. Bioassays for the testing of infectivity and virulence**

Parameters of baculovirus host range, infectivity, and virulence are tested by bioassays which measure the response (*e.g.* mortality) of test species ("subjects") to different doses of defined virus preparations ("stimulus"). The *in vivo* bioassay is the only means by which the combined effect of all factors determining the potency of a baculovirus preparation can be measured. It is also used as a very sensitive method of monitoring the fate of baculoviruses in the field (see C.28). The titration of occlusion bodies in the light microscope offers a convenient method to compare different virus preparations with respect to the concentration of their (potentially) active structures. In principle, any comparisons between two virus preparations (*e.g.* a test substance with a standard preparation) requires that the dose response curve for both have the same shape, *i.e.* the bioactive component of the preparations is essentially similar. An understanding of the basic biological and statistical concepts of bioassays is important to fully realise their potential for analysing baculovirus interactions with other organisms and for improving their application (Hughes and Wood, 1986).

To obtain statistically meaningful results with the desired precision bioassays have to be carefully designed with respect to the **number of subjects** challenged. Optimised strategies depending on previous knowledge and model assumptions have been described (e.g. Hughes and Wood, 1986). Bioassays determining the yes or no (quantal) response of larval death through virus infection result in dose response relationships with the median effective dose  $ED_{50}$  (or  $EC_{50}$  if expressed in virus particles per ml) and the slope of the response curve as characteristic parameters. In addition to counting dead individuals, response data may also include the determination of particle titers in the cadavers. If the time course of larval death is recorded (time-mortality assays), as conveniently done in one experimental approach, monitoring results yield median lethal time  $LT_{50}$  (continuous exposure to virus) or median survival time  $ST_{50}$  (one inoculum at start of experiment), respectively.

To obtain meaningful and reproducible results other conditions of bioassays must be carefully observed. With respect to the **test organism**, uniform instar stages of larvae are used and reared under identical conditions (temperature, light regime, diet, group size). The ability to hatch insect larvae reproducibly with minor premature deaths (e.g. by bacterial infections) is one major problem for testing a great number of different test species, as required for host spectrum analysis.

**Infection** is frequently performed by applying the virus preparation to the surface of the natural or artificial diet - or by incorporating virus suspensions to the components of the artificial diet. Depending on the experimental design, the test species are kept on inoculated material for the duration of the assay - or the regime includes a transfer to a noninoculated diet, in order to better control inoculum and infection time. A large variety of experimental protocols for controlled virus inoculation have been described. They all suffer from inaccuracies, to which the variation of feeding behaviour of the insects also contributes (Hughes and Wood, 1986). Direct administering of virus suspensions by microinjection into the mouth of larvae, or the "measured drop feeding" method are ways to improve control over dose and inoculation time (Kunimi and Fuxa, 1996). The incubation of eggs with virus suspensions to infect neonate larvae seems to be a method for a well controlled infection of large numbers of insects at an identical developmental stage. Some of the latter methods are time consuming and have other limits (size of larvae for injection) which prevent their widespread use. Experimental protocols in general have to seek a sensible compromise between accuracy and practicability. Modifications of the assays have to accommodate the habits and peculiarities of the test species.

Data interpretation follows model assumptions. If the only interest is an estimate of the median effective dose and no inferences with respect to the absolute number of virus particles can be made, the probit model may be used. It assumes a log-normal distribution of individual effective doses (IED) for each host which invariably induces the response. The model refers to mechanistic interpretation of chemical interactions and assumes some co-operativity to be necessary for infection. The necessity to overcome host defenses can be considered as an interpretation of such assumptions in light of present knowledge. However, unlike chemicals, replicating entities such as bacteria and viruses can act independently to produce a response. In these cases, and if the hosts are reasonably uniform in their susceptibility to the pathogen, the dose response curve can be described and interpreted more adequately by an exponential model. The model describes infection as a stochastic process in which the lethal dose is the same for all individuals whereas the response rates reflect the chance to receive this dose. Essentially, one infectious virus entering the replicative stage is able to manifest the response according to such a model. Predictions of quantitative features of the infection process according to the model can be tested experimentally and results do not conflict with its interpretation, stressing the general applicability. (Hughes *et al.*, 1984; Hughes and Wood, 1986; Ridout *et al.*, 1993).

The method of "*in vivo*-cloning" takes advantage from the capacity of single viruses to initiate a productive infection. Administering virus titers for infection far below the  $LD_{50}$  results in a relatively large fraction of surviving larvae. The fraction of larvae, which died from replication of a single

infectious unit can then be calculated (Huber and Hughes, 1984). This method, eventually used in two consecutive steps to enhance probability of the selection, is conveniently used to obtain genetically homogeneous virus preparations (Smith and Crook, 1988).

## **B. Survey of non-target effects**

The specificity of the interactions of baculoviruses with arthropod and the corresponding narrow range of species which are susceptible to productive infection by a particular virus, is the basis of their innocuousness for a large spectrum of nontarget organisms (Gröner, 1986). In the course of safety assessments toxicity and pathology studies have been performed on mammals, birds and other wildlife animals including beneficial insects such as the honeybee and silkworm. The studies with animals other than arthropods up to 1986 were extensively reviewed by Gröner (1986). In the following, the studies are only briefly summarised because they conclusively demonstrated the absence of any adverse effects.

- Toxicity studies on mammals with a variety of NPVs, using the spectrum of application routes as conventionally tested for chemical pesticides, never resulted in any indications of toxicity or pathogenicity using doses 10 to 100 times the per-acre field rate equated to a 70-kg man. Also, no indications of teratogenic or carcinogenic effects in mammals were found with challenges of NPVs (section B).
- No side effects on birds after oral application and on aquatic vertebrate and invertebrate animals could be observed. Such laboratory studies were supplemented by some extensive monitoring for pathological effects of wildlife birds and mammals after (aerial) applications of different NPVs.

Recently, toxic effects were observed with a larval test which was considered to be useful for assessing adverse effects of microbial pest control agents on nontarget bivalves because of its simplicity, precision, and sensitivity. Larvae of the coot clam *Mulinia lateralis* were challenged for 48 h during the straight hinged stage of development with the LdMNPV at a density of  $10^6$  OB/ml. Mortalities observed were significantly higher than those obtained with a heat killed control. Similar mortality was observed with a  $10^{-4}$  dilution of a commercial mosquito larvicide based on *Bacillus thuringiensis* ssp. *israelensis* (Bti). No effect was observed with a molluscidal strain of *Bacillus alvei* or a broad-host-range fungal insect pathogen, *Metarhizium anisopliae*. Sodium dodecyl sulfate and a watersoluble fraction of a fuel oil were tested as a reference for comparison (Gormly *et al.*, 1996).

- Consistent with their restriction of infectivity to the family or at least order of their original host, no infectivity or adverse effects on beneficial insects like pollinators (bees) have been observed. Baculovirus infection interferes with the multiplication of parasitoids within the same host. This interaction seems to be described most adequately as a competition for the same resource. No productive infection of the parasitoids is observed (see C.23/24).
- No genotoxic effect was observed by cytological studies after challenging mammals or cell cultures.

Similar studies with granuloviruses are smaller in number but gave the same results.

Small mammals or birds and also parasitoids feeding on insects infected by a baculovirus may take up and transport intact baculoviruses (*e.g.* in their digestive tract). Excretion of infective viruses may contribute to virus dispersal (C.26).

One experimental approach to use vectorised transport by honey bees as a nonintrusive means for virus dispersal has been field tested and indicates the perception of safety and non-target-innocuousness of baculoviruses. An applicator in a specifically designed substructure of a conventional beehive caused honey bees to take up (by surface contamination) and disseminate a talc formulation of HzSNPV into fields of *Trifolium incarnatum*. Increased HzSNPV induced mortality was observed in the clover fields foraged by the bees. A good persistence of baculovirus infectivity in honey was noted. An increased knowledge about the intersection of bee and target organism behaviour determining the virus transmission was considered to be essential, in order to further investigate the feasibility of the approach (Gross *et al.*, 1994b).

### C. Host range

The range of arthropods that can productively be infected by a given baculovirus can be regarded as its host range in a narrower sense. On one side, a limited host range is a feature of a rationally targeted biocontrol programme and an issue of biological safety. The control of defined pest species avoids side effects on the environment in general, and adverse effects on non-target insects including predatory and parasitoid species which provide additional control, in particular. On the other side, host range restrictions are also frequently regarded as an important application constraint if a complex of pest insects has to be managed. They determine limitations for the potential market volume of viral insecticides. Reports on particular host range testing and the selection strategies on virus strains for applications reflect this ambivalent view on host range restriction.

Among the approaches to relief application constraints for virus insecticides, prospects of modifying - and enhancing - host range by the use of molecular biology tools are being developed (Doyle *et al.*, 1990; Thiem, 1997). The realisation of this objective will challenge the validity of host range testing procedures and the interpretation of their results even more than the use of genetically modified viruses does in general. An increasing knowledge about the molecular interactions involved in host range determination and limitations is the basis for such an approach. In comparison to the use of selective strategies on natural viruses such a basis may enable a design of modifications for more targeted and predictable effects on virus phenotype (Miller and Lu, 1997; see section A.3.3).

The genetic information of the virus genome, conserving evolutionary processes for perpetuation of its existence in time, is realised in the sequence of processes during an infectious cycle typically resulting in the release of high numbers of progeny virus from larval carcasses (see section A.3.1). Abortive or persistent infections indicate potential alternative strategies for persistence. A productive infection requires the specific interaction of host components with virus structure and virus encoded functions within a temporal and local arrangement of “key-lock fittings” in order to overcome host barriers and defenses and to exploit and transform host functions for viral replication. Overall, these specificities correlate with phylogenetic relationships, and the systematics of baculoviruses seems to represent the common evolutionary origin and concerted evolution with their arthropod hosts to some degree.

In detail however, evolution - and the variation and distribution of “keys” and “lockers” - follow different pathways in virus populations and in sexually reproducing animals. Genome modifications by mutations and genetic exchange are governed by different mechanisms and constraints. Thus, the degree of adaptation of a virus strain or “species” to a set of host organisms providing the requirements for productive interactions (specificities) presently is not easily discernible from its systematic position (which is defined by the degree of homology of (parts of) his genome to other viruses). In the future, the molecular analysis of the determinants (genes) for virus-host interactions and of their variability in natural populations may provide more power of inference with respect to the host range of a particular virus.

So far, the testing with host species *in vivo* provides the relevant empirical basis for judgements about host ranges. Methods of data generation with respect to the following cannot follow harmonised and accepted internationally standardised procedures:

- The control of the homogeneity of the virus inoculum.
- The administration of the virus and the range of concentrations to be used.
- The spectrum of insect or other arthropod species and cell types to be included.
- The selection and control of end points (*e.g.* larval death, virus multiplication and yield, transcription of genes, DNA replication).

Parameters of influence on testing results include:

- The particular genotype of test species.
- The developmental stage of larvae during infection.
- The diet for host.
- Other conditions challenging the general stress tolerance of the test species (*e.g.* temperature, moisture).

Descriptions of a host range spectrum based on empirical testing will always suffer from ambiguities and incompleteness, due to apparent limitations in test species selection and experimental parameter variations. Studies of virus entry, DNA replication, transcription of genes and assembly of virus progeny together with the observation of cytopathic effects in cell cultures do substantially contribute to an understanding of host specificities at the level of molecular interactions (Miller and Lu, 1997). They complement but do not supplement, so far, *in vivo* testing of host range limitations (Danyluk and Maruniak, 1987; Castro *et al.*, 1997). It may be stressed again, that an understanding of mechanisms is a prerequisite to improve inferences from genotype to host specificity phenotype.

Host range and parameters of virulence may be correlated to a different extent. An increase in virulence may appear as a host range extension in conventional testing using fixed doses of virus challenge. However, the spectrum of susceptible host species may not have changed to any extent. A maintenance of the order and quantitative relations of infectivity parameters ( $LD_{50}$ s) would indicate an unchanged host range. A precise definition of experimental methods and end points clearly is a prerequisite for a common understanding of host range and virulence parameters.

The modification of virulence/host range by a selective procedure in the laboratory, cited by Federici and Maddox (1996) to exemplify a natural ability to manipulate host range, can be used to illustrate the importance of both, virulence and host range determinations. Martignoni and Iwai (1986a) used large doses of the MNPV of *Orygia pseudotsugata* (tussock moth, family: Lymantriidae) to sequentially infect *Trichoplusia ni* (cabbage looper, family: Noctuidae) over a period of 12 generations. In the course of these experiments, representing a selective pressure which in its extent will not occur in natural settings with any realistic probability, the virus adapted to the novel host, the cabbage looper. Low mortalities and minor levels of infection in most tissues at the beginning were converted to increasing levels of tissue infection and a complete adaptation of the virus to the novel host by the seventh generation. The passaged virus strain, if not selected from a heterogeneous inoculum, apparently accumulated some (set of) unknown mutation(s) and also had increased its

virulence for its original host, the tussock moth, by a factor of ten. This increase in virulence, however, would not in itself necessarily result in the expansion of the host range, as observed.

An overview of the host ranges of baculoviruses, as determined in cross infectivity studies in the laboratory is given by Gröner (1986). Results presented in tables are cited with the reservation that most studies did not control the identity with the inoculum virus after challenging of different hosts. Thus, the activation of a latent virus infection followed by its replication or the selective propagation of a minor contaminating virus type cannot be excluded in most cases. In general, NPVs infect only members of the genus, or in some cases the family, of their original host. A tendency that multiple embedded NPVs have a somewhat broader host range than SNPVs and an even more limited host range of granuloviruses was noted. Among the MNPVs, the prototype AcMNPV is known for its relatively broad host range and infectivity for more than ten families within the order Lepidoptera.

The identification of virus progeny by molecular methods is recommended and used as a standard tool for host range determinations in other publications as well. Predominantly a restricted range of infectivity is corroborated (Barber *et al.*, 1993). Comprehensive studies on a broad selection of potential host insects were induced by the necessity to generate an adequate background for respective studies with genetically modified baculoviruses (Doyle *et al.*, 1990; Doyle and Hirst, 1991). These studies resulted in the finding that MbMNPV is infective for both, butterflies and moths (Doyle and Hirst, 1991). MbMNPV of the cabbage moth *Mamestra brassicae* (Lepidoptera: Noctuidae) also infected the small tortoiseshell butterfly, *Aglais urticae*. However, a very high virus inoculum (106 OBs) was necessary for virus multiplication and larval death. MbMNPV also infects *Cynthia cardui* (painted lady) and *Vanessa indica* (Indian red admiral), also of the Nymphalidae, but does not seem to infect other butterflies of the families Satyridae, Lycaenidae, or Pieridae (Doyle *et al.*, 1990). The cross infection studies showed a somewhat broader host range of MbMNPV than previously demonstrated. Other baculoviruses are also infective for Nymphalid butterflies.

The (quantitative) interpretation of biotest data with respect to potential consequences from exposing natural populations, has not been investigated in any detail but could be regarded as a key area of judgements about baculovirus biosafety (Doyle *et al.*, 1990; Cory *et al.*, 1997). It is conceivable that the non-susceptibility at lower doses might normally prevent epizootics from being initiated from weak cross infectivities, *e.g.* after spray applications for biological control. But an improved potential of extrapolation from infectivity studies with respect to a potential of virus transmissions in heterologous populations is highly desirable, to more clearly define relevant borders of host range. Data from laboratory bioassays might be poor estimates for the prediction of nontarget impacts.

A multiple-embedded nucleopolyhedrovirus, AnfaMNPV, isolated from the celery looper, *Anagrapha falcipera* in central Missouri seems to have the broadest host range reported so far (Hostetter and Puttler, 1991). 31 susceptible species belong to ten lepidopteran families, and in addition to *Helicoverpa sp.*, *Heliothis sp.*, and *Spodoptera sp.* include important pests like pink bollworm (*Pectinophora gossypiella*), fall webworm (*Hyphantria cunea*), cutworms (Noctuidae), velvet bean caterpillar (*Anticarsia gemmatilis*), codling moth (*Cydia pomonella*), navel orangeworm (*Amelois transitella*), and the diamondback moth (*Plutella xylostella*). As compared to AcMNPV, known for its relatively broad host range, the virus demonstrated equal virulence for species differing in susceptibility for AcMNPV by one order of magnitude. Relatively narrow criteria were used for differentiation of susceptibility (max. 250 OB/mm<sup>2</sup> of diet). The list of nonsusceptible species as published (containing also Coleoptera and Diptera) might thus include some species which are susceptible, if challenged at higher doses. Propagation of virulent AfMNPV in tobacco hornworm (*Manduca sexta*) as untypical host demonstrated extensive possibilities for production in a variety of hosts and systems. Virulence against a great number of cosmopolitan economically important insect

species was considered an encouraging result in this report and the virus became the first baculovirus patented by the US Government Patent and Trademark Office.

#### **D. Variation of virulence and host range**

By describing interactions between biological entities, virulence and host range are prone to modification by natural variability and by a variety of external “environmental” factors. Mutagenesis, recombination, and the (selective) propagation in different hosts have been analysed as causes of shifts in host range and virulence of baculoviruses, in the laboratory (sections A.3.3; A.6-8.). The same mechanisms are acting in natural or managed environments, however to an extent unknown in any detail and responding to different selection conditions. In the following, emphasis is given to some factors modulating insect responses to viral challenges that have not been described in other sections.

##### ***External (environmental) factors modulating insect response to baculoviruses***

###### ***Chemicals***

An additive or synergistic effect of chemicals on baculovirus infectivity has been exploited for control strategies, for example, by the use of combinations with insecticides or the addition of particular radiation protectants (section A.6).

###### ***Host plants***

The plant material on which larvae are feeding may have a significant influence on their susceptibility for viral infections. Gypsy moths, feeding on *Quercus rubra* (red oak) or *Acer rubrum* (red maple) demonstrated a higher level of tolerance when infected with LdMNPV than larvae feeding on *Populus tremuloides* (quaking aspen) or *Pinus rigida* (pitch pine). The enhanced tolerance was correlated with an increased acidity and hydrolysable tannin content of leaf material (Keating and Yendol, 1987; Keating *et al.*, 1988). Different tolerances for *Spodoptera littoralis* NPV were also noted in the cotton leaf worm on castor bean, alfalfa, mulberry, cotton and potato. LD<sub>50</sub> values differed by a factor of about 3 between castor bean (the most “protective” feeding source) and potatoes, and the LT<sub>50</sub> was also reduced by more than 10% on the latter plant (Santiago-Alvarez and Ortiz-Garcia, 1992).

A different approach was followed by Rabindra *et al.* (1994), who investigated the influence of plant surface environments on the virulence of *Heliothis armigera* SNPV. The larvae of the American bollworm were challenged with identical (microscopically controlled) titers of virus suspensions which had been exposed to the surfaces of 5 host plants (chickpea (*Cicer arietinum*), pigeon pea [syn. *dhal*] *Cajanus cajan*), lablab bean (*Dolichos lablab*), sunflower, and cotton). Bioassays used the leaf dip method with shoots of chickpea as identical feeding material for the test species. The cotton leaf surface was the most detrimental for virus activity resulting in an increase of more than three orders of magnitude in the LC<sub>50</sub> value in comparison to lablab bean -exposed viruses (5 x 10<sup>6</sup> OB/ml and 1.4 x 10<sup>3</sup> OB/ml, respectively). Lablab bean even had some protective or stimulating effect on virulence in comparison to untreated NPV preparations (LC<sub>50</sub> = 8 x 10<sup>4</sup> OB/ml). Median lethal time measurements corresponded with these observations. A mortality time expanded by 25% after cotton leaf - exposure in comparison to lablab bean exposed virus was observed. This experimental approach was also used to determine the effect of adjuvants added to the virus preparations because the study together with others indicates the need to develop suitable formulations which could protect the virus from inactivating factors on plant surfaces.

These examples demonstrate the modulation of baculovirus virulence by the plant environment on which target insect larvae are feeding. This environment either indirectly alters insect response and tolerance, or interferes with the first steps of virus infection through variation of the milieu of the midgut lumen (e.g. pH), and/or directly modulates the specific activity (the probability of an OB in the light microscope to enter the replicative state) of virus preparations. An understanding of these kinds of interferences is valuable to improve formulations for insect control and to adapt methods of *in vivo* baculovirus production. It can also be used to reassess control strategies if applied to novel plant varieties (Beach and Todd, 1988). In an advanced stage of development of modelling the variability introduced by the respective plant canopy may be included. (Foster *et al.*, 1992).

#### *Population density*

A variety of environmental factors such as temperature, light, nutrition, and humidity may compromise the capability of insects to resist baculovirus infections (Briese, 1986). Among these, rearing density has a distinct effect on their susceptibility to viral infection. Together with other phenotypic modifications (development time, weight at moulting, degree of melanisation), enhanced susceptibility is observed as a reaction to the stress of high population density of lepidopteran larvae (Goulson and Cory, 1995b). This was interpreted as a consequence of the adaptive response of accelerated development at the expense of larval weight, which compromised the ability to express resistance functions. Interestingly, larvae which were reared singly exhibited many of the same characteristics, including enhanced susceptibility to virus infection. The benefit of spending resources for resistance development may be low, if a low density of conspecifics reduces infection risk. This phenotypic variation is among the factors modulating the relationship between virus density and disease transmission.

#### *Other infectious agents, parasitoids*

The exposition of insects with different infectious agents or parasitoids is a different kind of stress, eventually enhancing baculovirus induced mortality (C.24). Superinfection of insects with a latent or persistent infection may result in larval death due to the propagation of the latent virus (see A.4.6).

#### *Insect resistance and immunity*

The varying degree of tolerance of insect larvae for baculovirus infections at different instar stages, usually increasing with age and stage of development, is but one observation suggesting the existence of particular mechanisms of pathogen tolerance (Mikhailov *et al.*, 1992; Engelhard and Volkman, 1995). To assess the short-term and long-term effectivity of control strategies with baculovirus insecticides an understanding of the adaptive potential of insect populations to baculovirus infections is of paramount importance. In a review Briese gives an account on developmental and environmental factors affecting resistance (1986), the knowledge about defence mechanisms and the genetic factors conferring increased virus tolerance. Dominant and recessive autosomal genes or a multigene family may form the genetic basis of tolerance in insects. *E.g.* McIntosh and Ignoffo (1989) demonstrated that the resistance of *Helicoverpa subflexa* against *Helicoverpa zea* NPV (HzSNPV) appears to be controlled by a single non sex-linked gene.

Observations of markedly expressed virus resistance are restricted to laboratory observations of insect populations obtained under high artificial selective pressure of virus challenges, for example, by propagating the surviving fraction of infections with an LD<sub>50</sub> in several subsequent generations. Somewhat conflicting results with respect to the success of selection strategies may either be caused by the general potentials of the insect species under investigation or by the particular genotype of the

strain as most obvious reasons (e.g. Kaomini and Roush, 1988). It is a general objection against the predictive value of laboratory selections, that insect cultures in the laboratory represent a minor fraction of the genetic heterogeneity of natural populations. The lack of documented cases of resistance in field populations of insects under control of a virus insecticide may reflect their less frequent and extensive use in comparison to chemicals, which have induced an exponentially increasing rate of resistance developments in insect populations.

There is some circumstantial evidence that the spectrum of tolerance and respective gene frequencies in natural populations may have been biased by natural virus epizootics or the application of virus insecticides (Fuxa *et al.*, 1988). Laboratory selections in colonies of velvetbean caterpillar (*Anticarsia gemmatilis*) from the US and Brazil demonstrated the achievement of a significantly higher level (1000x) of resistance to AgMNPV after 13 - 15 generations in the Brazilian colonies than in the colony from Louisiana, the resistance of which levelled off at a ratio of 5x after 4 generations (Abot *et al.*, 1995). AgMNPV, is used for biological control of the velvetbean caterpillar in soybean on a large scale in Brazil (see A.6.4).

Alleviation of the selection pressure by virus challenges seems to favour more competitive sensitive wild-type genotypes of insects, which are superior in their reproduction without the virus selection pressure (Fuxa and Richter, 1989). Such observations correspond to a more general perception that some cost in reduced fitness has to be paid for the expression of disease resistance. This hypothesis however, has not been tested rigorously in many cases (Gemmill and Read, 1998). Genes for virus resistance confer pleiotropic phenotypic effects including increased susceptibility to chemical insecticides (Fuxa and Richter, 1990). The exclusive restriction to the oral application route of the increased virus tolerance and enhanced insecticide susceptibility phenotypes indicates a correlation of modifications to components of the midgut interfering with early stages of virus infection.

Using the genetically modified virus AcMNPV-hsp70/lacZ for the monitoring of virus activity by the lac-Z reporter gene, the physiological basis of resistance of *Helicoverpa zea* against AcMNPV was analysed by Washburn *et al.* (1996). The larval cells were actually very susceptible to AcMNPV infection, but infected cells were encapsulated by hemocytes and subsequently cleared from the midgut lumen. Cellular immune responses of larvae seem to be a significant factor in preventing the spread of the infection and a determinant of the functional host range of baculoviruses. Based on further experiments including chemical and biological immune suppression, it was suggested to use modified viruses to express immunosuppressive genes in order to compensate for this defence mechanism.

#### **4. Interactions with and Effects on Other Organisms in the Environment**

Direct effects of baculoviruses on other organisms in the environment are restricted to their host range, and most have been observed only in their original host or in the target species of biocontrol applications. Baculovirus infection interferes with other pathogens or parasitoids feeding on a shared host. Observations on the synergistic or additive effect of particular crossinfections with other pathogens as well as interferences with parasitoids have been summarised by Harper (1986). Section A 4.2 gives some account to the synergistic co-operation between some baculoviruses. Some of such pathogen combinations have been used in field experiments to improve control of pest species.

The analysis of the interference with parasitoid developments is used to assess the possibility of adverse effects on populations providing additional natural control of pest species. The observed detrimental effect on parasitoid development, fitness or reproductive success as well as an enhanced sensitivity of parasitised insect larvae for baculovirus infection can most adequately be described with

the interspecific competition for a limited resource (Cossentine and Lewis, 1988; Hochberg, 1991; Al Fazairy *et al.*, 1993; Nakai and Kunimi, 1997). The relative timings of baculovirus infection and parasitoid emergence determine the degree of interference to a large degree. No productive infection of parasitoids was observed but frequently a large number of viral occlusion bodies is present in their midgut lumen, indicating a potential mechanism of virus dispersal. A reduced level of a parasitoid population has been observed after aerial LdMNPV application (Webb *et al.*, 1989).

## 5. Ability to Form Survival Structures

The structure of baculovirus occlusion bodies provides for their potential to persist in the environment in a variety of abiotic and biotic conditions (A.4.1/4.7). This feature is described as a key factor dominating the dynamics of virus transmission and a prerequisite of baculovirus epizootiology by Evans (1986). Viability may extend for more than 40 years in forest soil. No special survival structures are formed.

## 6. Routes of Dissemination, Physical or Biological

### A. Physical dispersal

Transport and dispersal of baculoviruses by wind or water is considered to be of minor importance for the natural spread of baculovirus infections. Its contribution to mechanisms of primary dispersal in natural epizootics is not known in detail (Briese, 1986). In field experiments, testing the spatial spread of a baculovirus, ballooning of first instar larvae of the gypsy moth *Lymantria dispar* was a good predictor of viral spread in the first few weeks. The scale of spread and its lack of directionality at later times however, did not match the results of a mathematical modelling even when primary dispersal by ballooning and short distance larval dispersal with a high rate of disease transmission were included. Circumstantial evidence suggested that autonomous dispersal by wind of occlusion bodies did not significantly contribute to dispersal. Parasitoid vectoring of viruses was discussed as the potential relevant additional dispersal mechanism (whereas vectoring by other animals was not considered (Dwyer and Elkinton, 1995). Evidence for a dispersal of NPV from soil to pine foliage in dust was presented as a likely explanation for the observed distribution of diseased larval colonies near a forest road by Olofsson (1988).

Aerial dispersal is the predominant mechanism of distribution for insect control applications; techniques and timing have to consider meteorological conditions and other agricultural procedures like irrigation for optimal distribution (Young, 1990; Payne *et al.*, 1996).

Reminiscent of traditional testing procedures for the prediction of exposition rates with chemicals, the leaching behaviour of baculoviruses in natural soil cores and columns filled with soil or sand has been tested to evaluate eventual rates of exposition of the groundwater table. Consistently, some experiments demonstrated the retention of baculoviruses in soil to be comparable or superior to that of other Viruses (Polio virus and bacteriophage f2 of *Escherichia coli* were used for comparison). Soil with higher levels of organic matter was less efficient than sand for the reference viruses but not for the baculoviruses (NPVs and GVs). Results were independent from the particular composition of percolating water. Lysimeter studies with baculoviruses did not result in any observation of positive samples of leachate taken at a depth of 1.5 m during 7 months of monitoring. The concentration of viruses applied in this experiment resulted in a surface exposition 3 - 4 orders of magnitude above concentrations used in agricultural applications ( $10^{14}$  particles of a Granulovirus in 20 L of tap water applied to a surface of 0.8 m<sup>2</sup>). A good retention of baculoviruses by soils was concluded and this property was tentatively attributed to the particular protein envelope of virus particles consisting of polyhedrin and granulin, respectively (Lopez-Pila, 1988).

The behavioural shift of some insect larvae following baculovirus infection (*i.e.* the movement to higher and exposed positions on host plants: “Wipfelkrankheit”) effects the physical distribution of baculovirus particles following death. It might be an evolutionary adaptation of the virus to increase horizontal transmission, *e.g.* by contaminating more foliage following rainfall (Vasconcelos *et al.*, 1996a; Goulson, 1997).

### **B. Vectorised dispersal by diseased larvae, predators and parasitoids**

Virus infected larvae are an effective dispersal agent even before larval death and carcass lysis, which results in very high inoculum density. Viable virus particles are also dispersed in the environment through either defecation or regurgitation during late stages of infection (Vasconcelos, 1996). Cannibalism of infected larvae may be another possibility if it occurs at any relevant frequency. Transport and dispersal of virus particles also occurs by predators feeding on diseased larvae or by parasitoids ovipositioning on and developing within larvae (Briese, 1986; Boucias *et al.*, 1987; Vasconcelos *et al.*, 1996b). The viruses remain viable after passage of the alimentary canal of vertebrates and invertebrates, however to a widely divergent extent.

With respect to the speed and distance of the transfer of infectious viruses, the dispersal by birds deserves some special interest. Birds may take up infected or dead larvae directly or feed on non vertebrate or small vertebrate predators. Droppings may contain up to some 10<sup>7</sup> polyhedra. But the contribution of particular dispersal routes and mechanisms to virus epizootics is difficult to assess conclusively. Even a coincidence of novel areas with infected larvae and viruses in bird droppings is only circumstantial evidence of a significant contribution of avian transport to virus epizootics (Entwistle *et al.*, 1977; Buse, 1977; Cory *et al.*, 1988; Entwistle *et al.*, 1993). Novel baculovirus infections appearing in *Gilpinia hercyniae* (European spruce sawfly) in a previously unexposed geographical area at a time, before any virus could be monitored in avian droppings, suggests a different mode of dispersal (Buse, 1977). Even more recent experiments including trials with different virus applications at different locations and the identification of virus types in bird droppings by molecular restriction-hybridisation methods must close with the statement, that “the actual role of birds and many other biotic dispersal agents in the spread of baculoviruses and other micro-organisms remains to be demonstrated” (Entwistle *et al.*, 1993). Clearly, the knowledge of the intersection in space and time of insect behaviour and development and the ecological habit of particular bird species (including feeding - and defecation - behaviour and territoriality) must be included in such investigations.

Such intersections are also determinants of success for any approaches using insects as biotic dispersal agents for insect control. These experiments also can be regarded as a demonstration of a potential natural dispersal role of the engaged species (Biever *et al.*, 1982; Young and Yearian, 1990, 1992; Gross *et al.*, 1994b - see C.23.2). Any intentions to surmount the demonstrative character of these experiments in order to biologise application strategies for baculovirus insecticides would require the input of increased knowledge. It remains speculative whether such a manipulation of insect and baculovirus ecology for efficient pest control can be achieved in a practicable and effective manner.

## **7. Containment and Decontamination**

Special containment conditions in order to protect workers and the environment from baculoviruses are not usually required. The establishment of confinement measures is determined by the need to protect insect and cell cultures and the experiment from unintended infections. In part, depending on laboratory experiences with outbreaks of virosis in insectaries, the extension of technical installations and working procedures is adapted to these protection objectives. Small particle size and

persistence of baculoviruses represent a challenge for effective measures. The performance of work with baculoviruses has to follow the principles of good microbiological practice. Local separation of cell culture and insect work is a minimum requirement. To change laboratory coats when beginning or leaving work with baculoviruses seems to be advisable.

Heating can be used for decontamination of laboratory material and media. Baculoviruses are inactivated at temperatures and times significantly below conventional microbiological sterilisation conditions (Martignoni and Iwai, 1977). Sodium hypochlorite and formaldehyde can be used for chemical decontamination. Sodium dodecyl sulfate was also tested for egg surface sterilisation to prevent ex ovarial (vertical) transmission. A lower impact on moth rearing and sufficient effectiveness of inactivation were noted (Ilsley *et al.*, 1980).

No decontamination procedure seems to be recommendable for environmental contaminations, *e.g.* by spills from baculovirus insecticide containers. In fact, any procedure would compromise environmental quality to a greater extent than the baculovirus preparation. Soil decontamination by formaldehyde treatment as used after the first field trial with a genetically modified baculovirus or similar treatments will eventually be used according to the respective risk perception.

## **8. Description of Detection and Monitoring Techniques, Including Specificity, Sensitivity, and Reliability**

### **A. Detection**

Detection of baculoviruses in the environment is frequently performed by collecting (diseased or dead) larvae and making microscopic observations of stained baculoviruses. While this technique is adequate to monitor an infectious process in an insect population, other techniques are required to analyse the abundance and activity of infectious particles in the environment. Methods may consist of biotests by feeding target larvae with potentially exposed (plant) material. In order to quantify virus particles and to additionally determine their infectivity, virus particles are separated from the habitat matrix by washing or sonication. Virus particles collected by centrifugation are stained and counted by microscopy (Taverner and Connor, 1992; Carruthers *et al.*, 1988). Subsequent biotesting is then performed by applying an aqueous suspension to the insect diet.

Biotesting may amplify rather low concentrations of viruses, and does not seem to generally be inferior in its sensitivity in comparison to molecular methods which can be employed to directly detect baculovirus sequences by probe hybridisations or by amplifying specific sequences via PCR. The potential of these methods has not been evaluated for the monitoring of field trials in any significant extent. A method to detect and identify baculoviruses by a combination of PCR (amplifying a homologous region of the polyhedrin gene) and subsequent differentiation by restriction analysis has been described for 8 baculoviruses (De Moraes and Maruniak, 1997). However, PCR does not differentiate between viable and non-viable viruses.

### **B. Identification**

For the identification and differentiation of Baculovirus species/strains, the restriction analysis and/or probe hybridisations has become an established method (Trzebitzky *et al.*, 1988; Bensimon *et al.*, 1987; Doyle *et al.*, 1990; Barber *et al.*, 1993; Entwistle *et al.*, 1993; Hughes *et al.*, 1997). The feasibility of the approach for ecological investigations was conclusively demonstrated by Laitinen *et al.* (1996) who used heterogeneities in restriction endonuclease patterns among geographic isolates to assess the origin and spread of genotypes. The number of restriction enzymes used and the cutting frequency determine the degree of differentiation of divergent genotypes. A quantification of the

composition of heterogeneous mixtures is difficult to achieve by the analysis of banding heterogeneities and submolar bands. *In vivo* cloning of the genotypes of the mixture and quantification of their frequency would be a labour intensive solution.

In recent years, the PCR became an efficient tool with high sensitivity and specificity for the identification and diagnostics of micro-organisms. Primer specificities and reaction conditions determine the amplification of specific DNA fragments. De Moraes and Maruniak (1997) adopted this technique to amplify a highly conserved region of 575 bp within the polyhedrin gene of different NPVs with a specifically designed pair of primers. Restriction endonuclease digestion of the PCR product resulted in specific restriction patterns of the 8 analysed nucleopolyhedroviruses (*Anagrapha falcifera* NPV, AcMNPV, AgMNPV, BmNPV, and HzSNPV, OpMNPV, SfMNPV, and SeMNPV).

A similar approach was chosen to detect low levels of *Spodoptera littoralis* NPV from viral occlusion bodies and from infected host larvae and to differentiate between *S. littoralis* NPV and AcMNPV (Faktor and Raviv, 1996). These experiments demonstrate that PCR-based methods are useful for rapid identification and allow cost effective and sensitive monitoring of wild-type as well as genetically engineered baculoviruses.

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## APPENDIX

### Abbreviations

BV	budded virus/virion
<i>cat</i>	chloramphenicol acetyl transferase
EGT	ecdysteroid-UDP-glucose polyhedrovirus
ELISA	enzyme linked immunosorbent assay
FP	few polyhedra
GV	granulovirus
hr	homologous regions
IAP	inhibitor of apoptosis
ICTV	International Committee on Taxonomy of Viruses
IED	individual effective dose
LC <sub>50</sub>	lethal concentration (50%)
LD <sub>50</sub>	lethal dose (50%)
LT <sub>50</sub>	lethal time (50%)
MNPV	multiple nucleocapsid nucleopolyhedrovirus
NPV	nucleopolyhedrovirus
NOB	non-occluded baculovirus
OB	occlusion body
ODV	occlusion derived virus
ORF	open reading frame
PCR	polymerase chain reaction
PE	polyhedron envelope
p. i.	post-infection
SNPV	single nucleocapsid nucleopolyhedrovirus
TE	transposable element

## SECTION 2 PSEUDOMONAS

### I. General Introduction

This document presents information that is accepted in the scientific literature concerning the known characteristics of fluorescent members of the genus *Pseudomonas* (rRNA group I pseudomonads). Regulatory officials may find this information useful in evaluating and establishing the properties of environmental applications of biotechnology which involve those micro-organisms which are the focus of this document. Consequently, a wide range of information is provided without prescribing when the information would or would not be relevant to a specific risk assessment. This document represents a "snapshot" of current information that may potentially be relevant to such assessments. However, Member countries have not yet attempted to put together an exhaustive literature review on all aspects of these organisms.

The genus *Pseudomonas* may potentially be utilised in a number of different engineering applications. These include *in situ* applications such as groundwater reinjection, air sparging, and bioventing. They also include *ex situ* applications such as landfarming, slurry phase remediation, and biopiles. Many of the potential uses under development or envisioned for the genus *Pseudomonas* involve improvement of air, soil or water quality, or cleanup of otherwise intractable environmental contaminants.

In considering information that should be presented on this taxonomic grouping, the Task Group for Environmental Applications of Modern Biotechnology discussed the list of topics developed in the "Blue Book", *Recombinant DNA Safety Considerations* (OECD, 1986), and attempted to pare down that list to eliminate duplications, as well as those topics whose meaning is unclear, and to rearrange the presentation of the topics covered so they would be easier to understand and use (see Section III).

This effort at refining the exposition of safety considerations in the "Blue Book" for application to the genus *Pseudomonas* has also recognized the importance of a thorough understanding of the characteristics of the particular application for which these organisms will be used. Worker and other human exposures, and environmental exposures, will differ depending on the method of application. This knowledge is likely to affect the types of information on particular taxa that regulatory officials deem relevant in specific risk assessments. Group I pseudomonads are known to display a range of pathogenic and toxicological characteristics in regard to humans, animals and plants. However, even though some of the rRNA group I pseudomonads are known to exhibit pathogenic properties, exposures of and potential impacts on humans, animals and plants may be relatively limited in some circumstances, *e.g.* when the micro-organisms are used in bioreactors of various sorts that have suitable controls on liquid and gaseous emissions, or when other specific mitigation or containment measures are in place. The factors discussed in this document may, therefore, have varying levels of impact on individual risk assessments, depending upon how and where the particular micro-organisms are used, *i.e.* depending on the likely exposures presented by the application.

Given the breadth of information contained in this document, it is hoped that it will be useful not only to regulatory officials as a general guide and reference source, but also to industry and to scientists involved in research.

This document is a consensus document for environmental applications involving fluorescent members of the genus *Pseudomonas* (rRNA group I). Section II is an introduction to the genus *Pseudomonas* and to the species which are the subject of the document. The format of the information is described in Section III, and the information is presented in Section IV. Section V contains the References.

## II. Introduction to the Genus *Pseudomonas*

### 1. Taxonomy

*Pseudomonas* is part of a large, heterogeneous and ubiquitous group of micro-organisms generally referred to as pseudomonads. The pseudomonads are characterised as being highly metabolically versatile, bioactive, and prolific colonisers of surfaces. Pseudomonads are gram-negative, straight or slightly curved rods with polar flagella; they are chemo-organotrophs with a respiratory, non-fermentative type of metabolism and are usually catalase and oxidase-positive. The taxonomy of the group has been clarified using 16S ribosomal RNA sequence analysis (Table 4.4).

The genus *Pseudomonas* corresponds to rRNA group I (Table 4.4). The type species for the genus is *Pseudomonas aeruginosa*. Strains are metabolically diverse, as well as having the capacity for denitrification and arginine degradation under anaerobic conditions. *P. aeruginosa* has been studied in more detail than any other pseudomonad using genetic techniques. Physical and genetic chromosome maps have been described (Romling *et al.*, 1989; Ratnaningsih *et al.*, 1990).

### 2. Applications

Pseudomonads have been identified to be of importance in bioremediation as a result of their tremendous capacity for biodegradation. They also offer considerable promise in agronomic applications, since many strains are bioactive, fast-growing, prolific colonisers of plant surfaces and are able to suppress or out-compete pathogenic and other deleterious micro-organisms.

#### A. Pseudomonads as candidates for bioremediation

Nutritional versatility is exhibited widely amongst the pseudomonads. Combined with the presence or acquisition of catabolic plasmids by large numbers of strains, pseudomonads have the potential to mineralise a wide range of natural organic compounds, including aromatic hydrocarbons. This versatility allows for the rapid evolution of new metabolic pathways for the degradation of synthetic compounds (xenobiotics), leading to their complete oxidation and mineralisation. The complexity of the catabolic routes indicates sophisticated systems of regulation to control the expression and achieve the co-ordination of catabolic activities. Although the degradative pathways of pseudomonads vary considerably, the metabolic routes are convergent and lead to a limited number of common intermediates such as catechols. These represent key intermediates for aromatic compound degradation.

It is also anticipated that the nutritional versatility of pseudomonads and the application of molecular genetic techniques will be harnessed in the design of catabolic pathways for environmental purposes (Ensley, 1994; Timmis, 1994). For example, a *Pseudomonas* strain was recently isolated that can utilise TNT (2,4,6-trinitrotoluene) as a sole nitrogen source, producing toluene, aminotoluene and nitrotoluenes as end products. This organism was, however, unable to utilise toluene as a carbon source for growth. By introducing the entire toluene degradation pathway carried on the TOL plasmid pWWO-Km, an organism was produced that could potentially completely mineralise TNT (Ensley, 1994). Despite some of the TNT being completely mineralised, the formation of some dead-end metabolites by reduction of the nitrotoluenes to aminotoluenes remains a problem.

## B. Agronomic applications

Pseudomonads also have great potential in agronomic applications, since they are prolific colonisers of plant surfaces and represent a significant component of plant microflora. Furthermore, they have been identified to possess traits that make them suitable as agents for biological pest control (O'Sullivan and O'Gara, 1992). These traits include an ability to produce antimicrobial molecules (antibiotics, antifungals and siderophores) and a capacity to compete aggressively with other micro-organisms for niches and to exclude phytopathogens.

## III. General Considerations

### 1. Subject of the Document: Species Included and Taxonomic Considerations

#### A. Species included

The subject of this document is a subset of seven species within the genus *Pseudomonas*, most of which produce fluorescent pigments. Many members of this set have been, or are likely to be, employed in various biotechnological applications in the environment. The seven species are: *P. aeruginosa*, *P. chlororaphis* (including *P. aureofaciens*), *P. fluorescens* (including *P. marginalis*), *P. fragi*, *P. putida*, *P. syringae*, and *P. tolaasii*.

#### B. Taxonomic considerations

##### *The genus Pseudomonas*

Prior to 1973, *Pseudomonas* was seen as one large heterogeneous genus with members sharing a few phenotypic features. Palleroni *et al.* (1973) concluded that five groups approximating genera, which were established on the basis of rRNA sequence homologies (Table 4.4), appeared phylogenetically distant from each other. Though these groupings were confirmed through DNA hybridisation experiments (Johnson and Palleroni, 1989), it nonetheless took a decade to transform them into discrete taxonomic units based on both phenotypic and genotypic associations. The groupings now comprise units of larger than genus rank. Species once called *Pseudomonas* are now classified as members of at least a dozen genera found within the original five homology groups (Table 4.4; Yabuuchi and De Vos, 1995a; Yabuuchi and De Vos, 1995b). The genus *Pseudomonas* is now strictly confined to members of the rRNA group I (Table 4.4).

The members of this genus still represent a somewhat heterogeneous collection of bacteria, but they are far more closely allied to each other than they are to species formerly having the genus name *Pseudomonas*. The type species for the genus is *Pseudomonas aeruginosa*. Strains of *P. aeruginosa* can be isolated from many environmental substrates, and appear uniform in a number of diagnostic characters (Palleroni, 1992b). It can be argued that *P. fluorescens* is more "typical" of the genus than is *P. aeruginosa*, but due to the difficulty of establishing defining characteristics for *P. fluorescens*, *P. aeruginosa* remains the choice for the type species (Palleroni, 1992c).

Common characteristics of the genus *Pseudomonas*:

- A. Gram-negative.
- B. Rod-shaped (straight, asporogenous, 0.5-1.0 X 1.5-4.0 µm).
- C. Motile due to polar flagella.
- D. Oxidase-positive (except for *P. syringae*).
- E. Oxidative metabolism (mostly saccharolytic, some non-saccharolytic species, no gas).
- F. Formation from sugars).
- G. Chemo-organotrophs.

- H. Catalase-positive.
- I. Growth with acetate as sole carbon source, most non-fastidious, few require growth factors.
- J. NO<sub>3</sub> reduced to NO<sub>2</sub> or molecular N<sub>2</sub>.
- K. Accumulate longer-chained polyhydroxyalkanoates.
- L. Produce pigments.
- M. Indole-negative.

### *The “fluorescent” subgroup*

The seven species considered in this document [*P. aeruginosa*, *P. chlororaphis* (including *P. aureofaciens*), *P. fluorescens* (including *P. marginalis*), *P. fragi*, *P. putida*, *P. syringae*, and *P. tolaasii*] are considered to be closely related to each other except for *P. aeruginosa* and *P. syringae* (Molin and Ternström, 1986; Janse *et al.*, 1992). These seven species are considered as the fluorescent subgroup of the rRNA group I, although *P. fragi* includes non-fluorescent strains.

*P. fluorescens*, *P. putida* and *P. chlororaphis* are seen as forming a complex, intertwined by a continuum of transitional strains (Molin and Ternström, 1986; Barrett *et al.*, 1986). Complicating the classification scheme is the observation that both *P. fluorescens* and *P. putida* comprise several biovars, each of which may deserve species rank, but which are so interconnected that adequate methods have not been devised to clearly separate the member strains for each biovar. *P. chlororaphis*, which also encompasses the strains formerly called *P. aureofaciens* and *P. lundensis*, a recently described species, were once considered to belong to separate biovars of *P. fluorescens*.

Also closely associated with the *fluorescens-chlororaphis-putida* complex is *P. fragi*. This species has some fluorescent strains, but is primarily non-fluorescent. *P. fragi* is also a complex of different phenotypes, many of which are closely allied with some biovars of *P. fluorescens* and also could be misidentified as *P. putida* (Molin and Ternström, 1986).

*P. aeruginosa*, the type species and most clearly defined member of the genus, is seen as separate from the *fluorescens-chlororaphis-putida* complex.

*P. syringae* and *P. tolaasii* are pathogens in a group that also includes other pathogenic species (*e.g.* *P. cichorii* and *P. viridiflava*). However, *P. tolaasii* is an oxidase-positive mushroom pathogen related to, and potentially confused with, members of the *P. fluorescens* supercluster (Janse *et al.*, 1992). *P. syringae* is an oxidase-negative plant pathogen comprising many pathovars derived from taxa that previously had species rank (Palleroni, 1984).

### *Fluorescence*

Pigments often provide valuable diagnostic characters, since their production invariably correlates well with other group properties. Fluorescent pigments are produced abundantly in media with a low iron content; fluorescence varies from white to blue-green upon excitation with ultraviolet radiation. King’s medium B is frequently used for the isolation of pseudomonads, especially by plant pathologists (King *et al.*, 1954). Fluorescent species of *Pseudomonas* produce pyoverdinin and/or phenazine pigments. Pyoverdinin production is characteristic of most species. Palleroni (1984) indicates that *P. fluorescens* biovars II and V, along with *P. chlororaphis* and *P. putida* biovar B, have variable (11-89% positive) pyoverdinin production. Although positive pyocyanin production is diagnostic for *P. aeruginosa*, the reverse is not necessarily true.

Table 4.4 Phylogeny and current classification of the pseudomonads

Proteobacteria subclass	rRNA group	Original name	Current classification	Characteristics
Gamma	I	* <i>P. aeruginosa</i>	<i>Pseudomonas</i>	type species; opportunistic pathogen
		* <i>P. fluorescens</i>		fluorescent supercluster; oxidase positive, mostly fluorescent, saprophytic or opportunistic pathogens
		* <i>P. chlororaphis</i>		
		<i>P. lundensis</i>	* <i>P. chlororaphis</i>	name reclassified
		* <i>P. putida</i>		
		* <i>P. tolaasii</i>		mushroom pathogen
		<i>P. marginalis</i>	* <i>P. fluorescens</i>	name reclassified
		<i>P. aureofaciens</i>	* <i>P. chlororaphis</i>	name reclassified
		* <i>P. fragi</i>		some strains non-fluorescent
		* <i>P. syringae</i>		fluorescent, plant or mushroom pathogen. <i>P. syringae</i> and <i>P. viridiflava</i> are oxidase-negative. <i>P. syringae</i> comprises many pathovars
		<i>P. viridiflava</i>		
		<i>P. cichorii</i>		
		<i>P. agarici</i>		
		<i>P. asplenii</i>		
		<i>P. flavescent</i>		fluorescent
		<i>P. alcaligenes</i>		non-fluorescent
		<i>P. citronella</i>		
		<i>P. mendocina</i>		
		<i>P. oleovorans</i>		
		<i>P. pseudoalcaligenes</i>		
		<i>P. stutzeri</i>		
Gamma	V	<i>P. maltophilia</i>	<i>Stenotrophomonas maltophilia</i>	related to <i>Xanthomonas</i>
		<i>P. marina</i>	<i>Delia marina</i>	
Beta	III	<i>P. acidovorans</i>	<i>Comamonas</i>	
		<i>P. terrigena</i>		
		<i>P. testosteroni</i>		
		<i>P. avenae</i>	<i>Acidovorax</i>	facultatively autotrophic species (hydrogen pseudomonad)
		<i>P. delafieldii</i>		
		<i>P. facialis</i>		
		<i>P. flava</i>	<i>Hydrogenophaga</i>	facultatively autotrophic species (hydrogen pseudomonad)
		<i>P. palleroni</i>		
		<i>P. pseudoflava</i>		
		<i>P. taenispinalis</i>		
		<i>P. saccharophila</i>	<i>Pseudomonas</i>	
		<i>P. ruhlandii</i>	<i>Alcaligenes xylooxidans</i>	
Beta	II	<i>P. cepacia</i>	<i>Burkholderia</i>	heterogenous genus
		<i>P. caryophylli</i>		
		<i>P. gladioli</i>		
		<i>P. mallei</i>		
		<i>P. pickettii</i>		
		<i>P. pseudomallei</i>		

Proteobacteria subclass	rRNA group	Original name	Current classification	Characteristics
		<i>P. solancearum</i>		
Alpha	IV	<i>P. paucimobilis</i>	<i>Sphingomonas paucimobilis</i>	
		<i>P. carboxidovorans</i>	<i>Oligotropha carboxidovorans</i>	
		<i>P. aminovorans</i>	<i>Aminobacter aminovorans</i>	
		<i>P. mesophilica</i>	<i>Methylobacterium mesophilicum</i>	
		<i>P. sp.</i>	<i>Chelatobacter heintzii</i>	
		<i>P. compransoris</i>	<i>Zavarzinia compransoris</i>	
		<i>P. diminuta</i>	<i>Brevundimonas diminuta</i>	very distantly related to rRNA group I
		<i>P. vesicularis</i>	<i>Brevundimonas vesicularis</i>	

Source : Compiled from Palleroni, 1992b and 1992c; Molin and Ternström, 1986; Yabuuchi *et al.*, 1995a and 1995b, and Hildebrand *et al.*, 1994. The species which are the focus of this document are indicated with an asterisk.

#### Plasmid-encoded characteristics

Plasmid-encoded characteristics such as antibiotic resistance, chemical resistance and metabolic capabilities are important components of the pseudomonad genome. Some examples, for the species under consideration, are given in Table 4.5. Many if not most of these characteristics are strain-specific and of little value in terms of taxonomy and identification. An exception to this rule, however, is phage susceptibility. Plasmid-encoded phage susceptibility can be important in differentiating *P. syringae* pathovars when combined with biochemical testing, and *P. aeruginosa* isolates have been typed to the subspecies level using phage sensitivity.

### 4. Characteristics of the Organism Which Permit Identification and the Methods Used to Identify the Organism

#### A. General considerations

*P. aeruginosa* is distinct and readily distinguished from other members of the genus, and the pathovars of *P. syringae* can be distinguished by determination of their host range.

Distinction of the other five species in the group (*P. fluorescens*, *P. chlororaphis*, *P. putida*, *P. tolaasii*, *P. fragi*) from each other is not straightforward, and the expression “continuum” is frequently used to describe their inter-relationship. Most authors agree that current methods are generally inadequate to ensure proper placement of new isolates within the related species *P. fluorescens*, and *P. putida* and their biovars (Palleroni, 1992b; Christensen *et al.*, 1994; Barrett *et al.*, 1986). Except for its pathogenicity, *P. tolaasii* is difficult to distinguish from *P. fluorescens* (Janse *et al.*, 1992). *P. chlororaphis* is separable from *P. fluorescens* based on production of unique phenazine pigments (Palleroni, 1984), and has some distinctive substrate utilisation patterns (Barrett *et al.*, 1986), but is otherwise well within the boundaries of the fluorescent supercluster (e.g. Janse *et al.*, 1992). Finally, *P. fragi* shares many features with members of the fluorescent supercluster, but most strains are not fluorescent (Molin and Ternström, 1986).

## B. Methods used for identification and classification

### *Numerical taxonomy*

Numerical taxonomy has become the “traditional” method for classifying members of the genus *Pseudomonas*. This approach compares multiple features of the isolate, for which there is substantial discriminatory power, with a database of features of well-described members of the taxon. The accuracy of this type of approach will depend upon the quality and quantity of the data for strains comprising the reference database.

In order to achieve valid results, identical laboratory techniques need to be used for analysis of the isolate and the strains used to construct the reference database. The success of numerical taxonomy is also affected by the complexity of the relationships among the taxa being evaluated.

Use of a broad spectrum of substrates in numeric taxonomic evaluations has had some success for fluorescent species of *Pseudomonas* (e.g. Barrett *et al.*, 1986). These techniques have permitted some assignment of strains to species and biovars within the fluorescent supercluster (a term applied to all of the species and biovars of *P. fluorescens*, *P. putida* and their allies, Table 4.4). However, Molin and Ternström (1986) and Janse *et al.* (1992) both reported many unclassifiable strains among those they subjected to classical numeric taxonomic analyses.

Commercial suppliers have devised simplified, automated versions of this technique. Examples of commercial kits available for identification of *Pseudomonas* on the basis of carbon source utilisation patterns, and physiological and morphological characters, are the API20E (API, 200 Express Street, Plainview, New York 11803, USA; BioMérieux, F-69280 Marcy-L'Etoile, France) and the BIOLOG (BIOLOG Inc., Hayward, California, USA) systems. For these kits, the database for *Pseudomonas* is based on mainly clinical, not environmental, strains. As a result, the kits may fail to identify all environmental isolates.

Use of these kits requires experience. In addition, most of them are designed to determine the membership of the isolate within a taxon and not to distinguish strains within a species. That is, the test profile in most cases is not unique to a particular strain. So, in most cases, test profiles will not be sufficient to distinguish the isolate from other strains of the same species. If such a distinction is being made, it must be based on the detection of properties unique within the taxon.

Details of the test methodologies and profiles of the species can be found in Palleroni (1981; 1984; 1992c).

### *Genotypic approaches*

The current classification of the pseudomonads is based on rRNA homologies. The variable and conserved regions of the RNA molecule are both important for identification purposes. The conserved regions serve as targets for polymerase chain reaction (PCR) primer binding sites and universal hybridisation probes. The variable regions are the targets of the hybridisation probes and primers that are taxon-specific. Probes and PCR primers directed at diagnostic rRNA sequences have facilitated the classification of pseudomonads into the five rRNA groups (Table 4.4).

Strong selection pressure for the conservation of 16S and 23S rRNA molecular structure and sequence has meant that rRNA molecules are powerful evolutionary clocks for describing phylogenetic relationships between rRNA groups of pseudomonads. At present, however, they are unable to position individual strains into species groups. This is particularly true for the fluorescent rRNA group I pseudomonads (Christensen *et al.*, 1994). Using 23S rDNA methods, Christensen *et al.* (1994) found that “the method

failed to provide a basis for distinguishing between *P. fluorescens*, *P. chlororaphis*, and *P. putida* Biovar B and to differentiate among the biovars of *P. fluorescens*.” This study also showed that there did not seem to be a correspondence between taxonomies of this group based on 23S ribosomal sequences and from conventional numerical taxonomy. As pointed out by Janse *et al.* (1992), the large number of intermediate strains of all of these species shows “more variation than the present schemes (for classification) allow.”

Schleifer *et al.* (1992) describe several probes for the rapid identification of members of the genus *Pseudomonas*. A 360 bp fragment of a 23S rRNA gene derived from *P. aeruginosa* (Festl *et al.*, 1986) allowed differentiation of the eleven fluorescent and non-fluorescent group I species tested. A second probe was group-specific for *P. stutzeri*, *P. alcaligenes*, *P. pseudoalcaligenes* and *P. aeruginosa*. This probe comprises a 255 bp fragment of the 23S rDNA of *P. stutzeri* that is homologous to the bases 1366 to 1617 of *Escherichia coli* 23S rRNA. Both probes were tested by “dot-blot” hybridisation against genomic DNA bound to filters.

**Table 4.5 Examples of plasmids responsible for the metabolism of organic compounds or resistance to heavy metals in fluorescent *Pseudomonas* species**

Organism	Plasmid	Substrate	Reference
<i>P. putida</i>	CAM	camphor	Jacoby, 1975
	TOL	xylene, toluene	White & Dunn, 1978
	SAL	salicylate	Korfhagen <i>et al.</i> , 1978
	NAH	naphthalene	White & Dunn, 1978
	pRE4	isopropyl-benzene	Eaton & Timmis, 1986
	pEG	styrene	Bestetti <i>et al.</i> , 1984
	pCINNP	cinnamic acid	Andreoni & Bestetti, 1986
	pAC25	3-chloro-benzoic acid	Chatterjee & Chakrabarty, 1983
<i>P. fluorescens</i>	pQM1	mercury	Bale <i>et al.</i> , 1988
<i>P. syringae</i> pv. <i>tomato</i>	pPT23a; pPT23c	copper	Bender & Cooksey, 1986

Schleifer *et al.* (1992) used fluorescein or tetramethylrhodamine 5'-end-labelled sequences as probes to identify *Pseudomonas* species. The first probe (P72; 5'-TTCAGTACAAGATACCTA) differentiated *P. aeruginosa*, *P. alcaligenes* and *P. pseudo-alcaligenes* from the other group I species. A second probe, Ps (5'-GAAGGCTAGGCCAGC), identified all species except *P. putida*. An oligonucleotide specific for the *P. putida* sequence, 5'-GAAGGUUAGGCCAGC, allowed differentiation of *P. putida*, and a mixed probe (*i.e.* both oligonucleotides) allowed detection of all species of *Pseudomonas*.

#### Polymerase chain reaction (PCR)

*P. aeruginosa* strains can be identified by PCR-based amplification of the 16S-23S rDNA internal transcribed spacer region with specific primers (Tyler *et al.*, 1995).

#### Other biomarkers

Biomarkers such as fatty acid methyl esters (FAMES) have been used widely for the identification of bacterial species (Thompson *et al.*, 1993b). A commercial identification system, the Microbial Identification System (MIDI, Newark, New Jersey, USA), offers an extensive database of strains, including many pseudomonad strains, to which the fatty acid profile of an unknown isolate may be compared. The libraries of strains contain well-described clinical, environmental and plant-pathogenic strains. Taxis system provides an identification at the species level and a diagnostic profile; however, it is unlikely to identify all environmental isolates, since many have not been described before. Whole cell fatty acid analysis was tested as a method to discriminate between members of the *Pseudomonas* fluorescent

supercluster (Janse *et al.*, 1992). This analysis resulted in recognition of a large supercluster that included most *P. fluorescens* and related strains (*P. chlororaphis*, *P. putida* and *P. tolaasii*). In the supercluster there were no separate clusters discriminating biovars of *P. fluorescens*, the other related species, or strains received as *P. marginalis* (a name formerly applied to plant-pathogenic members of the supercluster). Thus, the resolution of this technique appears to have limitations.

Diagnostic profiles for micro-organisms may also be obtained using polyacrylamide electrophoresis of whole cell protein extracts, or DNA fingerprints produced via restriction endonuclease digestion of genomic DNA. Pseudomonads have genomes that are rich in GC DNA bases. Enzymes like *SpeI* that cut at sites with a high AT base composition will digest the DNA at only a few sites, producing large fragments which may be separated and analysed using pulse field gel electrophoresis.

The species of rRNA group I synthesize a ubiquinone with nine isoprene units (Q-9) in the side chain, whereas members of rRNA groups II, III, and V contain Q-8, and those of rRNA group IV a Q-10 (Oyaizu and Komgata, 1983).

Polyamine patterns are of similar utility: rRNA group I species have a high putrescine and spermidine content, rRNA group II and III species have 2-hydroxyputrescine and a high content of putrescine, rRNA group IV species only contain significant amounts of spermidine and sym-homospermidine, and rRNA group V species are characterised by high concentrations of cadaverine and spermidine (Busse and Auling, 1988; Auling, 1992; Yang *et al.*, 1993).

In summary, the results of any of the methods of identification described above are only as good as the database of strains and isolates to which they are referenced. There are numerous techniques that, if applied at the optimum taxonomic level, may prove useful in identifying *Pseudomonas* and its species. Ribosomal RNA sequencing seems useful at the genus or higher level, and methods like fatty acid analysis can work at the strain and isolate level.

### 3. Information on the Reproductive Cycle (Sexual/ Asexual)

*Pseudomonas* species reproduce by cell growth and binary cell division.

### 4. Biological Features and Environmental Conditions Which Affect Survival, Reproduction, Growth, Multiplication or Dissemination

#### A. General considerations

*Pseudomonas* species are efficient saprophytic chemo-organotrophs which grow at neutral pH and at temperatures in the mesophilic range (optimal growth between 20° and 45°C). Some species will grow at 4°C (*P. fluorescens*, *P. putida*) but not at the elevated temperature of 41°C. Optimal temperatures are 25-30°C for *P. fluorescens*, *P. putida* and *P. syringae*, 30°C for *P. chlororaphis*, and 37°C for *P. aeruginosa*. Most, if not all, species fail to grow under acid conditions (< pH 4.5) (Palleroni, 1984).

*Pseudomonas* species thrive under moist conditions in soil (particularly in association with plants), and in sewage sediments and the aquatic environments. Environmental conditions which will affect their growth include nutrient availability, moisture, temperature, predation, competition, UV irradiation, oxygen availability, salinity, and the presence of inhibitory or toxic compounds. As nutritional demands are modest, pseudomonads can survive and multiply for months in moist environments such as tap water, sink drains, etc. (Palleroni, 1981; Bergen, 1981).

Competition and competitive niche exclusion are likely to limit the growth of introduced pseudomonad inoculants. Competitors are likely to include closely related pseudomonads and other bacteria able to compete for the same ecological niches with similar nutritional requirements (Lindow, 1992).

A number of environmental conditions may affect the dissemination of *Pseudomonas* species including surface water runoff, wind currents, and rain splash. It is likely that insects and other animals may also serve as vectors for dispersal. For example, *P. aeruginosa* can inhabit part of the normal gut or skin microflora of humans and animals. Thus dissemination would be associated with these vectors.

#### **B. *P. aeruginosa***

*P. aeruginosa* is capable of growing in conditions of extremely low nutrient content (Palleroni 1984). The species was found to survive and proliferate in water for up to 100 days or longer (Warburton *et al.*, 1994). Conditions of high humidity and temperature (80-90% humidity, 27°C) favoured the colonisation of lettuce and bean plants (Green *et al.*, 1974).

#### **C. *P. chlororaphis***

*P. aureofaciens* (*P. chlororaphis*) is an important coloniser of the rhizosphere and phyllosphere of plants (Thompson *et al.*, 1993a; Legard *et al.*, 1994). Kluepfel *et al.* (1991b) reported the colonisation of wheat roots in a field release of a recombinant *P. aureofaciens* (*lacZY*). This population of *P. aureofaciens* reached a maximum of  $2 \times 10^6$  cfu/g root two weeks after inoculation and declined steadily to reach a level below detection ( $<100$  cfu/g root) by 38 weeks post-inoculation. Angle *et al.* (1995) found that inoculations of recombinant *P. aureofaciens* (*lacZY*) survived approximately twice as long in wheat rhizosphere as in non-rhizosphere bulk soil.

England *et al.* (1993) compared the survival and respiratory activity of *P. aureofaciens* in sterile and non-sterile loam and sandy loam soil microcosms. Recovery of *P. aureofaciens* was greater in sterile than non-sterile soils. Respiratory activity was higher in sandy loam soil than in loam soil, but soil type had no effect on survival.

The growth of *P. aureofaciens* in the spermosphere of seed-inoculated sugarbeets exhibited long lag phases (8-12 h) and their populations increased mainly between 12 and 24 hours (Fukui *et al.*, 1994). The doubling time during the exponential growth phases was 2-3 h (Fukui *et al.*, 1994).

#### **D. *P. fluorescens***

*P. fluorescens* is commonly found inhabiting plant rhizosphere or phyllosphere environments. The plant rhizosphere provides an environment in which the species may show improved survival and growth. *P. fluorescens* distributed homogeneously in soil can result in significantly higher numbers in the rhizosphere of young wheat plants than in non-rhizosphere soil (Trevors *et al.*, 1990).

The survival of *P. fluorescens* is affected by a variety of abiotic and biotic factors. Rattray *et al.* (1993) found that temperature and soil bulk density had a significant effect on lux-marked *P. fluorescens* colonisation of wheat rhizospheres. The greatest rates of colonisation occurred at the highest temperature (22°C) and lower bulk density (0.82 g/cm<sup>3</sup>), and 100-fold higher numbers were found in the ectorrhizosphere than in the endorhizosphere. Van Elsas *et al.* (1991) found *P. fluorescens* cells were able to withstand low temperatures, and could survive better at 4°C than at 15 or 27°C following introduction into natural soil, possibly due to an inhibition of the activity of the indigenous microflora. Van Elsas *et al.* (1986) found that *P. fluorescens* cell numbers declined slowly in both silt loam and loamy sand, but

survival was better in the silt loam. Heijnen *et al.* (1993) found that *P. fluorescens* survived better in unplanted soils in the presence of bentonite clay. Stutz *et al.* (1989) demonstrated that survival of *P. fluorescens* in vermiculite clay was better than in montmorillonite, which was better than in illite.

Van Elsas *et al.* (1992) found *P. fluorescens* R2f survived above  $10^7$  cfu/g dry soil for up to 84 days in Ede loamy sand microcosms when encapsulated in alginate with skim milk and bentonite clay, while free cells declined below  $10^5$  cells/g dry soil after 21 days. Vandenhove *et al.* (1991) studied the survival of *P. fluorescens* inocula of different physiological stages in soil. Introduction of a late exponential phase inoculum into soil brought about a lower death rate compared to exponential or stationary phase inocula.

Handley and Webster (1993) studied the effect of relative humidity (RH at 20, 40, 60, and 80%) on airborne survival of *P. fluorescens* indoors. They found that *P. fluorescens*, suspended in distilled water, survived best at mid humidities and least at 80% relative humidity.

Boelens *et al.* (1994) and Bowers and Parke (1993) determined that motility of *P. fluorescens* did not affect its spread through soil. A non-motile mutant strain promoted plant growth and colonised roots as effectively as the motile strain. Water flow rates were more important than motility for dispersal through soil and rhizospheres. Knudsen (1989) developed a mathematical model for predicting aerial dispersal of bacteria during environmental release which predicted off-site dispersal patterns that were in qualitative agreement with results from a field release of a genetically engineered *P. fluorescens* in California.

Thompson *et al.* (1992) studied dissemination of *P. fluorescens* by placing bacterial populations on apple or pear pollen in the entrances of hives of honey bees. In a pear orchard, 72% of the flowers within 7.6 m of the hive were colonised with *P. fluorescens* eight days after the start of the study.

#### ***E. P. fragi***

*P. fragi* is commonly found on refrigerated meat and dairy products (Jay, 1992). Psychrotrophs such as *P. fragi* generally have a lower metabolic rate than mesophiles (lower  $Q_{10}$  for the same substrate) and have membranes that transport solutes more efficiently (Jay, 1992). In addition, there is a correlation between the maximum growth temperature and the temperature at which respiratory enzymes are destroyed in psychrotrophs. Nashif and Nelson (1953) reported that extracellular lipase synthesis in *P. fragi* was inactivated at 30°C. The lipase of *P. fragi* is reported active at temperatures as low as -29°C (Alford and Pierce, 1961).

*P. fragi* has the ability to colonise stainless steel surfaces in food processing establishments to form "biofilms" (Hood and Zottola, 1995); attachment may involve a polysaccharide and protein matrix surrounding the cells (Herald and Zottola, 1989). Attachment of *P. fragi* to stainless steel surfaces occurred in 0.5 h at 25°C and in 2 h at 4°C through the development of attachment fibrils (Stone and Zottola, 1985).

#### ***F. P. putida***

A variety of environmental factors can affect the survival of *P. putida*. For example, plant rhizospheres can provide an environment for improved survival. Gamliel and Katan (1992) studied the chemotaxis response of *P. putida* towards seed exudates and germinating tomato seeds and suggested this may contribute to its rapid establishment in plant rhizospheres. Temperature is also an important factor. Hartel *et al.* (1994) found that *P. putida* (*lacZY*) declined from about  $10^8$  to  $10^3$  cfu/g of soil after 35 days at 35°C, while it did not survive after three days at 40°C.

Macnaughton *et al.* (1992), using pLV1013 as a marker plasmid in *P. putida* PaW8, investigated the effect of soil texture on survival and found that introduced bacteria survived better in soils with higher clay

content. Compeau *et al.* (1988) studied survival of *P. fluorescens* and *P. putida* strains in sterile and non-sterile soil. Colonisation of sterile soil by one strain precluded normal colonisation of the second added strain and suggested there were limited sites for colonisation of *Pseudomonas* species in these soils.

Lynch (1990) found that *P. putida* WCS 358 survived in distilled water (without substrate input) for over a year. It was suggested that this could result from the utilisation of dead cells within the population, and from viable cells having a very low maintenance energy requirement in a state of arrested metabolism.

Madsen and Alexander (1982) found that cells of *P. putida* were not transported below 2.7 cm in moist soil in the absence of some transporting agent or in the presence of developing plant roots. Percolating water and a burrowing earthworm enhanced the vertical transport of *P. putida*.

### G. *P. syringae*

Foliar plant pathogens such as *P. syringae* are generally not adapted to survival in the soil (Lindow *et al.*, 1988). However, *P. syringae* has been isolated from plant debris in the soil and can overwinter in temperate climates (Hirano and Upper, 1983). *P. syringae* pv. *syringae* R32 have pili that function as adhesions anchoring the cell to the surface of plants, thereby enhancing epiphytic colonisation. Wild-type bacteria became virtually resistant to displacement by rinsing within one day after inoculation, whereas non-piliated mutants were only partially resistant within three days (Suoniemi *et al.*, 1995).

*P. syringae* metabolises a broad range of substrates, thus demonstrating flexibility in nutrient utilisation (Hirano and Upper, 1990). Criteria for viability of cells have been modified as the result of starvation experiments with *P. syringae*; it was determined that respiration of acetate and glycerol were more accurate determinants of viability than respiration of succinate (Cabral, 1995). The use of bactericides in agriculture (streptomycin and copper) has resulted in selection for strains resistant to these compounds; the resistance is often encoded on plasmids (Cooksey, 1990).

Plant-pathogenic strains grow to larger population sizes on susceptible plant hosts than on resistant ones (Stadt and Saettler, 1981), and therefore pathovars of *P. syringae* will grow to greater numbers on their respective hosts than on non-hosts. The presence of free water may be the most important factor contributing to the increase in population of *P. syringae* pv. *syringae* to infectious levels on bean leaves (Hirano and Upper, 1983; 1990; Beattie and Lindow, 1994). Immediately after rainfall, there is an initial decrease in population as bacteria are washed off the leaf surface, followed by a rapid increase in the population within 12 to 24 hours. Ambient temperature appears to have little effect on field populations of *P. syringae* pv. *syringae* on leaves but the age of annual crops does have an effect, with many more cells found on older leaves than on younger ones (Hirano and Upper, 1990; Jacques *et al.*, 1995). *P. syringae* pv. *savastanoi* causes tumors on olive and oleander by producing the plant growth regulators indoleacetic acid (IAA) and cytokinins following infection; mutants deficient in IAA production grew as well as the wild type in culture and on plants, but the wild type reached a higher population density and maintained its maximum density at least nine weeks longer than the mutant populations.

Rainfall plays an important role in redistributing *P. syringae* within the plant canopy by washing bacteria from upper leaves onto lower ones, and by allowing individual bacterial cells to move using their flagella and find protected micro-sites on the surface of the leaf (Beattie and Lindow, 1994). Rainfall efficiently removed bacteria from foliar surfaces, but most of the cells were washed onto the soil; only a small portion were washed a relatively short distance from the source (Butterworth and McCartney, 1991).

*P. syringae* is also dispersed on seeds (Hirano and Upper, 1983). When cells of *P. syringae* were applied as a spray to plots, an exponential decrease in numbers of cells was observed; some cells were detected 9.1 m downwind within 20 minutes of the spray application. When applied to oat plants (a non-

host), viable cells could be detected for up to 16 days and were detected on plants up to 27 m downwind. In contrast to the plants, viable cells could not be detected in the upper layers of soil after two days (Lindow *et al.*, 1988).

#### **H. *P. tolaasii***

In the production of commercial mushrooms, *P. tolaasii* probably survives between crops on structural surfaces, in debris, and on equipment. It can be moved readily from one crop to another on the hands of pickers, on materials or equipment used in harvesting, and by insects, mites, water droplets and mushroom spores.

Conditions of high relative humidity and surface wetness encourage the expression of symptoms of brown blotch, an important mushroom disease, caused by *P. tolaasii*. Dispersal of the micro-organisms occurs readily upon watering once the disease is established (Howard *et al.*, 1994).

### **5. Behaviour in Simulated Natural Environments Such as Microcosms, Growth Rooms, Greenhouses, Insectaries, etc.**

#### **A. *P. aeruginosa***

In a study by Sturman *et al.* (1994), the growth rate of *P. aeruginosa* appeared to be important in determining interspecies competition within packed-bed bioreactors filled with diatomaceous earth pellets.

#### **B. *P. chlororaphis***

Angle *et al.* (1995) found that an intact soil core microcosm closely simulated survival results obtained from a field release of a recombinant *P. aureofaciens* (*lacZY*). The strain of *P. aureofaciens* survived approximately 63 days in the bulk soil microcosm and 96 days in the rhizosphere microcosm.

#### **C. *P. fluorescens***

Binnerup *et al.* (1993) found that kanamycin-resistant cells of *P. fluorescens* DF57-3 (Tn5 modified) inoculated in soil microcosms rapidly lost their culturability, as defined by visible colony formation on Kings B agar supplemented with kanamycin. After 40 days, only 0.02 to 0.35% of the initial inoculum was culturable. It was determined that about 20% of the initial inoculum represented viable, but non-culturable cells.

Compeau *et al.* (1988) studied survival of *P. fluorescens* and *P. putida* in sterile and non-sterile soil. Colonisation of sterile soil by one strain precluded normal colonisation of the second added strain and suggested there are limited sites for colonisation of *Pseudomonas* species in these soils. Similarly, Al-Achi *et al.* (1991) found that, when introduced as pairs into irradiated, sterile soils, a *P. fluorescens* strain prevented optimum colonisation by a *P. putida* strain. The addition of *P. putida* to sterile soil already populated by *P. fluorescens* impeded growth of *P. putida* in that soil. However, adding *P. fluorescens* to soil populated by *P. putida* did not prevent growth of *P. fluorescens* and caused a decrease in *P. putida*. These results suggested that there was competition for similar niches in soils, and that *P. fluorescens* was the more competitive species studied.

#### **D. *P. fragi***

No information was found regarding the behaviour of this species in microcosms, greenhouses, insectaries, etc.

### E. *P. putida*

Winstanley *et al.* (1993) studied the survival of *P. putida*, with an xy/E marker insert, in soil and lake water microcosms. When released into these microcosms, populations of the marked *P. putida* had a steady decline with little or no apparent division of cells. The rate of decline of *P. putida* in soil microcosms was significantly greater at 35% than at 50% field capacity water content, indicating that water content of the soil had an effect on survival. Similarly, Iwasaki *et al.* (1993; 1994) reported that the density of *P. putida* decreased rapidly to less than  $10^2$  to  $10^3$  level within five days in lake water and soil microcosms. The survival was influenced by protozoa density, light intensity, and soil water content. The addition of *P. putida* ( $10^1$  cfu/ml) into natural water and soil had no effect on the density of indigenous micro-organisms and enzyme activities.

Williams *et al.* (1992) studied the fate and effects of *P. putida* PPO200 genetically engineered for both nalidixic acid and kanamycin resistance in freshwater and marine microcosms. The freshwater microcosm contained fish (*Poecilia latipinna*), annelid worms (*Tubifex tubifex*), snails (*Gyraulus* sp.), freshwater mussels (*Margaritifera margaritifera*), freshwater shrimps (*Palaemonetes kadiakensis*), and plants (*Elodea canadensis*). The marine microcosm contained fish (*Cyprinodon variegatus*), sea anenomae (*Bunodosoma californica*), snails (*Turbo fluctuosus*), oysters (*Crassostrea gigas*), estuarine shrimp (*Palaemonetes pugio*), shorefly larvae (*Ephydra* sp.), and plants (*Salicornia bigelovii*). *P. putida* could be detected in the tissues of some non-target organisms, *i.e.* the bacterium survived. However, gross signs, survival, and the histological study of control and exposed non-target organisms indicated that there were no adverse effects.

Doyle *et al.* (1991) and Short *et al.* (1991) observed reductions in CO<sub>2</sub> evolution and the number of fungal propagules, as well as the enhancement of dehydrogenase activity in soil amended with 2,4-dichlorophenoxyacetate (2,4-D) and inoculated with *P. putida* PPO301(pRO103) genetically engineered to degrade 2,4-D. These unanticipated effects were not observed: (a) in uninoculated soil; (b) when the homologous, plasmidless parent *P. putida* PPO301 was inoculated; or (c) in the presence of the genetically engineered *P. putida* when 2,4-D was not added. Moreover, the effects were not predictable from the phenotype of this genetically engineered *P. putida*. While long-term, statistically significant differences were detected in some microbial populations and processes, the majority of the differences were transient.

The effects of *P. putida*, on nitrogen transformations and nitrogen-transforming microbial populations were studied in a soil perfusion system by Jones *et al.* (1991). Neither the genetically engineered strain nor its homologous plasmidless host had a significant effect on ammonification, nitrification or denitrification in the soil, or on the population dynamics of the micro-organisms responsible for these processes.

### F. *P. syringae*

Wendtpothoff *et al.* (1994) monitored the fate of a genetically engineered strain of *P. syringae* applied to the leaves of bush beans in a planted soil microcosm. *P. syringae* established on the bean leaves at between  $5 \times 10^3$  and  $4 \times 10^6$  cfu/gm<sup>-1</sup> fresh weight. During senescence of the bean plants, the strain was no longer detectable by selective cultivation and subsequent colony hybridisation.

Significant differences within *P. syringae* strain MF714R were detected when the bacterium was cultured on agar or in broth or collected from colonised leaves and subsequently inoculated onto greenhouse-grown plants in growth chambers or in the field or onto field grown plants. Bacterial cells cultured in liquid medium survived the least well after inoculation under all conditions, whereas cells cultured on solid media exhibited the highest percent survival and desiccation tolerance in the growth chamber but survived less well in the field than did cells harvested from plants. Cells harvested from plants

and inoculated onto plants in the field usually had the highest percent survival, started to increase in numbers earlier, and reached a higher number than did cells cultured *in vitro* (Wilson and Lindow, 1993a).

Wilson and Lindow (1993b) indicated that greenhouse-grown plants support larger epiphytic populations of an inoculated strain of *P. syringae* than do field-grown plants.

#### ***G. P. tolaasii***

No information was found regarding the behaviour of this species in microcosms, greenhouses, insectaries, etc.

### **6. History of Use (Examples of Environmental Applications of the Organism and Information Derived from These Examples)**

#### **A. General considerations**

Pseudomonads have been identified to be of importance in bioremediation as a result of their metabolic versatility. This metabolic versatility, and the ability to acquire additional versatility via plasmids, provides the potential for the rapid evolution of novel metabolic ability in *Pseudomonas* species. Examples of useful, or potentially useful, environmental applications of *Pseudomonas* isolates are given in Table 4.6. Some pseudomonad species have been introduced into the environment in bioremediation studies and have provided valuable information pertaining to characteristics such as survival. For example, Thiem *et al.* (1994) injected *Pseudomonas* sp. strain B13, a chlorobenzoate degrader, into a subsurface aquifer and found they could detect the strain 14.5 months after its environmental introduction.

Pseudomonads also have great potential in agronomic applications, since they are prolific colonisers of plant surfaces and represent a significant component of plant microflora. For example, they have been identified to possess traits that make them suitable as agents for biological pest control (O'Sullivan and O'Gara, 1992). These include an ability to produce antimicrobial molecules (antibiotics, antifungals and siderophores) and a capacity to aggressively compete with other micro-organisms for niches and exclude phytopathogens. The possibility of the environmental application of strains to minimise frost damage on crop plants has also been investigated (Lindow and Panopoulos, 1988).

#### **B. *P. aeruginosa***

Some strains of *P. aeruginosa* have been shown to produce biosurfactants which have potential uses in bioremediation for washing hydrocarbons from soil (Van Dyke *et al.*, 1993). Jain *et al.* (1992) found that biosurfactants produced by *P. aeruginosa*, when added to soil, significantly enhanced the degradation of tetradecane, hexadecane and pristane.

Degradation of pentachlorophenol by *P. aeruginosa* has been investigated in shake-cultures. The bacteria were able to completely degrade pentachlorophenol up to 800 mg/l in six days with glucose as a co-substrate (Premalatha and Rajakumar, 1994). *P. aeruginosa* has also been found to degrade styrene in a continuous reactor at a rate of 293 mg g<sup>-1</sup>h<sup>-1</sup>. This could be applied to the industrial treatment of waste gas or polluted water (El Aalam *et al.*, 1993).

#### **C. *P. chlororaphis***

A strain of *P. chlororaphis*, genetically engineered to contain the *lacZY* genes, was introduced into the environment in a field trial in the United States in 1987, and its behaviour compared to the non-engineered strain (Kluepfel *et al.*, 1991a, b, c). The non-engineered strain increased in number for two weeks, then declined to at or near the detection limit by 31 weeks.

**Table 4.6** Examples of fluorescent species of *Pseudomonas* reported to have been used, or to have potential use, for bioremediation

Species	Strain	Target chemical	Reference
<i>P. aeruginosa</i>	JB2	Halogenated benzoic acids	Hickey & Focht, 1990
	PaK1	Polyaromatic hydrocarbons	Kiyohara <i>et al.</i> , 1994
<i>P. fluorescens</i>	PHK	Phthalate	Pujar & Ribbons, 1985
		Dimethylphenol	Busse <i>et al.</i> , 1989
		Isopropylbenzene	Busse <i>et al.</i> , 1989
<i>P. putida</i>		Methyl-benzoates	Galli <i>et al.</i> , 1992
		Naphthalene sulphonic acid	Zurrer <i>et al.</i> , 1987
		Dimethylphenol	Busse <i>et al.</i> , 1989
	OUS82	Polyaromatic hydrocarbons	Kiyohara <i>et al.</i> , 1994
	G7	Polyaromatic hydrocarbons	Kiyohara <i>et al.</i> , 1994

**D. *P. fluorescens***

*P. fluorescens* has been genetically engineered and used in a number of experimental field studies, including the environmental introduction of a Tn-5 marked strain in the Netherlands in 1986 and other studies in the United States with strains engineered by deletion of the ice gene, and by introduction of *lacZY* marker genes (Wilson and Lindow, 1993b). De Leij *et al.* (1995) found that field releases of a genetically engineered *P. fluorescens*, and the unmodified wild-type strain, resulted in significant but transient perturbations of some of the culturable components of the indigenous microbial communities that inhabited the rhizosphere and phylloplane of spring wheat, but no significant perturbations of the indigenous culturable microbial populations in non-rhizosphere soil were found. The release of both of these bacteria had no obvious effect on plant growth and plant health, and the observed microbial perturbations were considered minor.

*P. fluorescens* can produce large and unusual proteins that are a key component of bacterial ice nuclei (Warren, 1987). Warren (1987) and Lindow and Panopoulos (1988) reviewed the practical applications connected to ice nucleation, including snow-making and the use of ice nucleation gene-deletion strains to generate biological control agents for minimizing frost damage to plants. A naturally occurring *P. fluorescens* strain, A506, has been registered commercially for the control of frost injury of pear (Wilson and Lindow, 1993b).

*P. fluorescens* is also one of the more common bacterial species that has been used for the control of diseases in the phyllosphere of plants, and a naturally occurring strain of this species has been registered for the commercial control of fire blight on pear (Wilson and Lindow, 1993b). Hatai and Willoughby (1988) detected *P. fluorescens* and *Saprolegnia parasitica* in rainbow trout lesions and found that *P. fluorescens* could strongly inhibit the growth of the fungus. It was suggested that *P. fluorescens*, or an antibiotic derived from it, might be used in biological control of saprolegniasis.

Snyman *et al.* (1993) found that *P. fluorescens* genetically engineered to produce the insecticidal toxin from *Bacillus thuringiensis* was toxic to *Eldana saccharina*. An LC<sub>50</sub> of 1.86 mg freeze-dried bacterial powder/ml of insect diet was calculated, and it successfully reduced sugarcane boring.

*P. fluorescens* has been shown to have the ability to degrade a wide variety of compounds, including: 3-chlorobenzoic acid (Fava *et al.*, 1993); naphthalene, phenanthrene, fluorene and fluoranthene (Weissenfels *et al.*, 1990); chlorinated aliphatic hydrocarbons (Vandenbergh and Kunka, 1988); styrene (Baggi *et al.*, 1983); and pure hydrocarbons and crude oil (Janiyani *et al.*, 1993). *P. fluorescens* can also be used in biosensor applications. For example, the recombinant *P. fluorescens* strain HK9, which lights up in the presence of contaminants such as PAHs (due to the insertion of lux genes), allows easy detection of bioavailable fractions of pollutants in soils and sediments (King *et al.*, 1990).

#### **E. *P. fragi***

No information was found on the use of *P. fragi* in environmental applications.

#### **F. *P. putida***

*P. putida* is capable of eliminating phytopathogenic micro-organisms and stimulating plant growth (Vancura, 1988; Kloepper *et al.*, 1988; Freitas and Germida, 1990). *P. putida* is also capable of degrading many unusual compounds by means of enzymatic systems encoded in plasmids. Chemicals degraded include polychlorinated biphenyls (PCBs) (Boyle *et al.*, 1993; Lajoie *et al.*, 1994); trichloroethylene (TCE) (Fujita *et al.*, 1995); acetonitrile and sodium cyanide (Babu *et al.*, 1994). *P. putida* has also shown the ability to remediate non-ionic sewage (Turkovskaya *et al.*, 1993), pulp mill waste (black liquor) (Jain *et al.*, 1993), waste gases using a biofilter (Zilli *et al.*, 1993), electroplating effluent with high concentrations of copper (Cu(II)) (Wong *et al.*, 1993), and high-sulphur coal (Khalid and Aleem, 1991).

#### **G. *P. syringae***

Lindow *et al.* (1988) monitored the fate of a strain of *P. syringae* in experimental field trials in the United States. They found an exponential decrease in numbers of viable cells deposited at increasing distances from sprayed field plots. The relative rate of survival of cells sprayed directly on plants was more than ten times higher than that of cells dispersed through the air to similar adjacent plants.

Use of *P. syringae* has been proposed to enhance snowmaking and to delay frost damage in plants (Lindow, 1983; Wilson and Lindow, 1993b). *P. syringae* has also been shown to incorporate aluminium, chromium and manganese, so the bioremediation of sites contaminated with these chemicals may be a potential use (Alaoukaty *et al.*, 1992).

#### **H. *P. tolaasii***

No information was found on the use of *P. tolaasii* in environmental applications.

### **7. Characterisation of the Genome (e.g. Presence of Large Plasmids, Insertion Sequences) and Stability of These Characteristics**

Members of the genus *Pseudomonas* are known for their metabolic versatility. They are capable of degrading many recalcitrant xenobiotics due to their ability to recruit new genes and alter the expression of existing ones. An understanding of the relative chromosomal position of relevant genes, the diversity of mobile genetic elements found within this genus, and the role these mobile genetic elements play in the stability and metabolic adaptation of individual isolates, can be helpful for regulatory assessments.

The chromosomes of *P. putida* and *P. aeruginosa* have been described in detail by Holloway and Morgan (1986) and by Ratnaningsih *et al.* (1990), Romling *et al.* (1989) and Holloway *et al.* (1994). Holloway *et al.* (1990a) provide genetic maps of these two species, which are useful in locating the relative positions of important genes and provide a good summary of other chromosomal and extrachromosomal

features. The sizes of the chromosomes for *P. putida* and *P. aeruginosa* vary from approximately 4,400 to 5,400 kb, with *P. aeruginosa* strain PAO having a genome size (5,400 kb) significantly larger than the 4,700 kb *E. coli* chromosome. Analysis of the distribution of chromosomal genes in pseudomonads shows that those involved in biosynthesis are not contiguous as with the enterobacteria. The genes for catabolic functions tend to be clustered on the chromosome, but are also not contiguous. Many catabolic functions are located on plasmids (e.g. Table 4.5); these genes, such as TOL (toluene degradation) and NAH (naphthalene degradation), tend to be contiguous. This genome configuration allows for many diverse substrains within a species, each adapted to a particular environment.

*Pseudomonas* species contain a large variety of plasmids, insertion sequences, and transposons. The diversity of plasmids involved in degradation of organic compounds, drug resistance, and phytopathogenicity is indicated in Tables 5.4, 5.7 and 5.10. Insertion sequences (IS elements) and transposons are mobile within the genome of gram-negative bacteria, and can act as new promoters or as terminators, causing polar mutations. If two IS elements are located near each other in the appropriate orientation, they can be transposed to a second genome as a unit along with any intervening genes.

These three classes of mobile genetic elements (plasmids, insertion sequences, and transposons) can potentially interact within the same isolate, causing shifts in the positions of key catabolic genes. An example is the NAH plasmid naphthalene degradative genes, which are nested within a defective but mobilizable transposon on the plasmid (Tsuda and Iino, 1990). Such shifts can result in a variable stability for some traits. For example, *P. syringae* pv. *savastoni* mutations causing IAA deficiency were identified to have resulted from the action of two IS elements. In another case, a 150 kb plasmid (able to integrate into the chromosome) from *P. syringae* pv. *phaseolicola*, when excised from the chromosome, resulted in the formation of a series of plasmids that either contained chromosomal DNA or were deletion mutants of the plasmid. These events were associated with a common repeated sequence (RS) (Coplin, 1989). In a reverse situation, components of the TOL plasmid have been shown to integrate into both the *P. putida* and *P. aeruginosa* chromosomes (Holloway *et al.*, 1990b), thereby potentially stabilizing degradative genes in the genome of the isolates.

Besides affecting the stability of certain traits, mobile genetic elements allow pseudomonads to recruit new genes from replicons such as plasmids, which can lead to new metabolic capabilities. Specific examples have been given by Chakrabarty (1995) of *P. putida*'s ability to recruit new degradative genes on a transposable element. These new genes allow the organism to degrade new chemicals without the need to evolve completely new degradative pathways. This species has been able to acquire the genes needed to degrade 3-chlorobenzoate to the intermediate protocatechuate, which then is further degraded by resident chromosomal genes. In a similar fashion, the same species has been able to degrade phenol by acquisition of two genes, *pheA* and *pheB*, whose products can convert phenol to intermediates which are metabolized by a chromosomally-encoded *ortho* pathway (Chakrabarty, 1995). A transposon-like mobile element encoding a dehalogenase function has also been recently described in *P. putida* (Thomas *et al.*, 1992). In the well-characterised *P. putida* mt-2 plasmid pWW0, the TOL-degradative enzymes are encoded on a 56 kb transposon which is itself part of a 70 kb transposon (Tsuda *et al.*, 1989), giving rise to a family of TOL plasmids (Assinder and Williams, 1990). In addition to acquisition of degradative genes, pseudomonads can also acquire genes whose products aid in waste degradation.

The chlorosis-inducing phytotoxin coronatine, produced by *P. syringae* pvs. *tomato* and *atropurpurea* is plasmid encoded (Coplin, 1989). Other toxins (e.g. phaseolotoxin, syringomycin and tabtoxin) have been shown to be chromosomally encoded. *P. syringae* pv. *savastoni* produces abnormal growths due to an imbalance of cytokinin and auxin plant hormones. The genes for their biosynthesis are plasmid encoded in oleander, but not olive pathovars. The majority of *P. solanacearum* strains contain a large (700-1000 kb) megaplasmid that contains genes for host range and pathogenicity.

## 8. Genetic Transfer Capability

The ability of pseudomonads to develop new metabolic pathway capabilities is often dependent on an isolate's ability to acquire DNA from other bacteria, which is then integrated into the genome in a manner dependent on the organism's environment. The three common systems for gene transfer in bacteria, namely conjugation, transduction and transformation, have been observed among members of the genus *Pseudomonas*. All three gene transfer mechanisms have been observed under laboratory and natural conditions. Gene transfer by all three mechanisms is affected by biological factors such as the nature and host range of the mobile genetic element, its transfer frequency, the concentrations of recipient and donor organisms, and the presence of other organisms which prey on donors and recipients. Abiotic factors such as temperature, moisture, and the presence of physical substrates which allow survival and/or gene transfer also affect the transfer frequency.

Even if the DNA is transferred to a new recipient, it may not be expressed. Sayre and Miller (1990) provide a detailed summary of factors associated with transposons and plasmids, the donors and recipients, and other biotic and abiotic conditions which affect gene transfer rates.

### A. Conjugation

The acquisition of genetic material via conjugative plasmids represents an important evolutionary mechanism in the production of strains resistant to antibiotics and heavy metals, and with the ability to mineralise xenobiotics in selective environments. Gene transfer events may even affect the pathology of certain phytopathogens. Changes in cultivar-specificity and a loss of ability to produce fluorescent pigments of *P. syringae* pv. *pisi* were found to result upon the acquisition of IncP1 replicons such as plasmid RP4. Curing the RP4 plasmid from the strain maintained the new phenotype (Moulton *et al.*, 1993). Walter *et al.* (1987) developed a combined mating technique to measure the conjugal transfer potential of conjugative plasmids that uses four different standard mating techniques (colony cross streak, broth mating, combined spread plate, and membrane filtration), since no one technique worked best for the tested combinations of plasmids and recipients.

Conjugation between pseudomonads has been detected in both soil and aquatic environments. The transfer of conjugative plasmids has been demonstrated to occur between pseudomonads in a number of non-rhizosphere and rhizosphere soil environments, both in microcosms and *in situ* (van Elsas *et al.*, 1988; Trevors and Berg, 1989; Lilley *et al.*, 1994).

Transfer frequencies were found to be enhanced by two orders of magnitude, that is, up to  $10^{-2}$  per recipient organism, on the rhizoplane of sugarbeet *in situ* (Lilley *et al.*, 1994). Soil components (such as clay, silt, organic matter and plant roots) provide excellent surfaces for the cell-to-cell contact required for bacterial conjugation (Trevors and Berg, 1989; Stotzky *et al.*, 1991). In wheat plant root (van Elsas *et al.*, 1988) and sugarbeet (Lilley *et al.*, 1994) conjugation studies, survival of the donor and recipient, as well as frequency of plasmid transfer, decreased with increasing distance from the plant root. Transfer frequencies are also affected by soil moisture, with frequencies for R-plasmid transfer between *E. coli* isolates shown to be optimal at 60 to 80% soil moisture holding capacity (Trevors and Starodub, 1987). Conjugal transfer of broad host range plasmids between *P. aeruginosa* donor and recipient strains in lake water has been observed to occur at a lower rate in the presence of the natural microbial community (O'Morchoe *et al.*, 1988). Plasmids incapable of conjugation themselves have been shown to be mobilised from a laboratory strain of *E. coli* in a laboratory-scale wastewater treatment facility by mobilizer and recipient *E. coli* strains of both laboratory and wastewater origin (Mancini *et al.*, 1987).

The TOL plasmid pWWO can be transferred to other micro-organisms, and its catabolic functions for the metabolism of alkylbenzoates are expressed in a limited number of gram-negative bacteria, including

members of the rRNA group I pseudomonads and *E. coli* (Ramos-Gonzalez *et al.*, 1991). Transfer of the recombinant plasmid to *Erwinia chrysanthemi* was observed, but transconjugants failed to grow on alkylbenzoates because they lost catabolic functions. Pseudomonads belonging to rRNA groups II, III, and IV, *Acinetobacter calcoaceticus*, and *Alcaligenes* sp. could not act as recipients for TOL, either because the plasmid was not transferred or because it was not stably maintained. Under optimal laboratory conditions, the frequency of transfer of pWWO from *P. putida* as a donor to pseudomonads belonging to rRNA group I was on the order of  $1$  to  $10^{-2}$  transconjugants per recipient, whereas the frequency of intergeneric transfer ranged from  $10^{-3}$  to  $10^{-7}$  transconjugants per recipient. Intra-species, but not inter-species transfer of TOL in soils has been reported (Ramos *et al.*, 1991), but it was affected by the type of soil used, the initial inoculum size, and the presence of chemicals that could affect the survival of the donor or recipient bacteria (Ramos-Gonzalez *et al.*, 1991).

The *P. putida* TOL plasmid pWWO and the wide host range RP4 plasmid are able to mediate chromosomal mobilisation in the canonical unidirectional way (*i.e.* from donor to recipient cells) and bi-directionally [*i.e.* donor to recipient to donor (retrotransfer)] (Lejeune and Mergeay, 1980; Mergeay *et al.*, 1987; Top *et al.*, 1992; Ramos-Gonzales *et al.*, 1994). Transconjugants are recipient cells that have received DNA from donor cells, whereas retrotransconjugants are donor bacteria that have received DNA from a recipient. The TOL plasmid pWWO and the pRP4 plasmid are able to directly mobilise and retromobilise a chromosomal marker integrated into the chromosome of the other *Pseudomonas* strains, and this process probably involves a single conjugational event. The rate of retrotransfer (as well as direct transfer) of chromosomal markers is influenced by the location of the marker on the chromosome, and it ranges from  $10^{-3}$  to less than  $10^{-8}$  retrotransconjugants per donor (transconjugants per recipient). The mobilised DNA is incorporated into the chromosome of the retrotransconjugants (transconjugants) in a process that seems to occur through recombination of highly homologous flanking regions. No interspecific mobilisation of the chromosomal marker in matings involving *P. putida* and the closely related *P. fluorescens* was observed.

It seems clear that pseudomonads can acquire plasmids from other bacteria in the environment. This premise is supported by the array of plasmids that have been recovered from members of *Pseudomonas*, some of which are listed in Tables 5.5, 5.7 and 5.10. The boundaries to gene transfer events are illustrated by plasmid RP4, originally isolated in *P. aeruginosa*, which has been shown to be transmissible to all gram-negative bacteria tested (Riley, 1989). *E. coli* has been shown to transfer plasmid-borne genetic information to over 40 genera (Stotzky *et al.*, 1991). Direct evidence of pseudomonad isolate acquisition of plasmids from other bacteria in the environment is also available: Bale *et al.* (1988) showed that an introduced *P. putida* recipient acquired mercury resistance plasmids from an intact lotic epilithic bacterial community at frequencies up to  $3.75 \times 10^{-6}$  per recipient.

## B. Transduction/bacteriophage mediated gene transfer

Two characteristics of a bacteriophage (phage) which are important in determining its ability to broadly distribute DNA were summarised in Sayre and Miller (1990). First, the host range of most phages is restricted to one species or a small number of related taxa, although broad host range phages such as phages P1 and Mu are known. Second, phages which undergo specialised transduction are likely to transfer chromosomal genes which are in close proximity to the phage integration site, while generalised transducing phages can transfer any of the bacterial genome's sequences with approximately equal frequency.

Many different lytic and temperate phages have been identified in *Pseudomonas*, and the morphological diversity among phages is at least as great as for any other bacterial genera. Transduction by temperate phage of *P. aeruginosa* chromosomal DNA has been demonstrated in fresh water microcosms

(Morrison *et al.*, 1979; Saye *et al.*, 1987; 1990) and the phylloplane of bean and soy bean plants (Kidambi *et al.*, 1994).

*P. aeruginosa* has been frequently reported as subject to lysogeny, the process by which the phage chromosome becomes integrated into the bacterial host chromosome and is stably replicated with it, as a prophage. Lysogeny may lead to increased fitness of bacterial strains in the natural environment, by increasing the size and flexibility of the gene pool available to natural populations of bacteria via horizontal gene transfer. Approximately 45% of *Pseudomonas* field isolates tested positive in colony hybridisations when probed with phage isolated from the same area (Miller *et al.*, 1990a). The prophage appears to contribute a major source of phage in the natural environment. In addition to mediating the transfer of genetic material within and between species, the induction of certain prophages results in transposition and mutagenesis events within the host genome.

### C. Transformation

Both chromosomal and plasmid DNA are subject to natural transformation in the environment, a natural physiological process which is different from the artificial transformation techniques used in the laboratory (Stewart, 1990). In order for transformation of a cell to result in expression of the new DNA sequence, DNA must: 1) be excreted or lost from a donor cell; 2) persist in the environment; 3) be present in sufficient concentrations for efficient transformation to occur; 4) come in contact with a recipient cell which is naturally competent to receive the donor DNA; 5) be able to evade any recipient cell defences which degrade foreign DNA; and 6) integrate into a stably-maintained replicon in the recipient. Marine and soil environments have been shown to contain biologically significant levels of dissolved DNA (Paul *et al.*, 1987; Lorenz *et al.*, 1988). Soil environments offer protection from nuclease digestion for chromosomal and plasmid DNA (otherwise available for transformation) when bound to clay and sediment matter (Lorenz and Wackernagel, 1991; Romanowski *et al.*, 1991; Khanna and Stotzky, 1992).

Natural transformation was found for *P. stutzeri*, *P. mendocina*, *P. alcaligenes* and *P. pseudoalcaligenes*, but not for *P. aeruginosa*, *P. putida*, *P. fluorescens*, and *P. syringae* strains (Carlson *et al.*, 1983).

## IV. Human Health Considerations

### 1. Diseases caused and mechanism of pathogenicity including invasiveness and virulence

#### *General considerations*

Included amongst the rRNA group I fluorescent pseudomonads are species pathogenic to humans. Those that do cause infection are generally opportunists, promoting a variety of conditions ranging from endocarditis to dermatitis (Pollack, 1992; Artenstein and Cross, 1993; Berger *et al.* 1995; Jarvis and Skipper, 1994). Individuals most at risk from *Pseudomonas* infection are the immunocompromised, in particular individuals with AIDS, patients with cystic fibrosis, and those suffering major trauma or burns (Artenstein and Cross, 1993; Neu, 1985; Bodey *et al.*, 1983; Moayyedi *et al.*, 1994; Schuster and Norris, 1994).

It should be stressed that only some of the Pseudomonads have been implicated in human disease and that proponents should not base assessment criteria on *P. aeruginosa*, the most problematic member of the genus. Proponents should also be aware that not all strains of a given species have been shown to promote disease, and that assessment criteria should be based upon the strain being reported rather than the species as a whole.

*P. aeruginosa*

The predominant pseudomonad isolated from clinical sources is *P. aeruginosa*. Evaluation of the pathogens causing nosocomial infections in hospitals in the United States (Jarvis and Martone, 1992) indicated that *P. aeruginosa* was the fourth most common pathogen isolated (10.1%) in hospital-wide surveillance and the most common (12.4%) in intensive care units. In more recent surveys, *P. aeruginosa* was found to be the fifth most common pathogen (9%) (Emori and Gaynes, 1993) and increased to 29% in intensive care facilities [European Prevalence of Infection in Intensive Care Study (EPIC) (Spencer, 1994)]. The ability of *P. aeruginosa* to persist in a number of hospital disinfectants and pharmaceuticals (Dominik *et al.*, 1995; Gilardi, 1991) and in sanitary facilities (Bobhammer *et al.*, 1996; Döring *et al.*, 1991; Zembruska-Sadkowska *et al.*, 1995) probably contributes to its prevalence in the hospital environment. *P. aeruginosa* causes a wide range of syndromes, involving nearly all body systems, that vary from sub-acute to chronic (Artenstein and Cross, 1993; Pollack, 1992).

Bacteremia associated with *P. aeruginosa* is restricted mainly to immunocompromised individuals with significant underlying disease (Artenstein and Cross, 1993; Askamit, 1993; Spencer, 1994). Symptoms are indistinguishable from gram-negative sepsis caused by other bacteria (Pollack, 1993). Factors associated with bacteremia include malignancy, transplants (solid organ, bone marrow), diabetes, cirrhosis of the liver, renal failure, burns, trauma, intravenous drug abuse, corticosteroid therapy, cytotoxic chemotherapy, cardiopulmonary resuscitation, immunoglobulin deficiency, AIDS, broad spectrum antibiotics, and invasive instrumentation (IV catheters, endoscopes, mechanical ventilation, etc.) (Askamit, 1993; Artenstein and Cross, 1993; Dropulic *et al.*, 1995; Mallolas *et al.*, 1990; Nensey *et al.*, 1990). Major entry portals for *P. aeruginosa* bacteremia appear to be following infection (or colonisation) of the respiratory tract (Gallagher and Watanakunakorn, 1990; Artenstein and Cross, 1993) and the genitourinary tract (Gallagher and Watanakunakorn, 1990; Aksamit, 1993).

*P. aeruginosa* septicemia is primarily a condition of debilitated, immunocompromised adults and of infants. It is usually acquired in hospital, with prior broad-spectrum antibiotic therapy as a predisposing factor (Bodey *et al.*, 1983; Richet *et al.*, 1989), although community-acquired infections in apparently healthy children (Ros, 1989) and adults (Ishihara *et al.*, 1995) have been reported. Mortality is high in immunocompromised patients (up to 50%) (Artenstein and Cross, 1993; Bisbe *et al.*, 1988), with a better prognosis in "normal healthy" individuals (Ishihara *et al.*, 1995).

Endocarditis due to *P. aeruginosa* has been associated with two major predisposing factors (Artenstein and Cross, 1993): the use of prosthetic heart valves and the use of illicit parenteral drugs. In drug abusers, endocarditis usually occurs in the right side of the heart and is sub-acute, although a complication, septic pulmonary emboli involving the tricuspid valve, can occur (Pollack, 1992).

Nosocomial pneumonia is the second most common hospital-acquired infection in the United States (Aksamit, 1993; Emori and Gaynes, 1993). *P. aeruginosa* is the most prevalent etiological agent for both poly- and mono-microbial pneumonia (Emori and Gaynes, 1993). Community-acquired pneumonia has also been attributed to this organism; its occurrence is rare (Artenstein and Cross, 1993), but it is more frequently recognised in AIDS patients (Dropulic *et al.*, 1995; Schuster and Norris, 1994). Colonisation of the oropharyngeal and/or the upper gastrointestinal tracts is an important precursor to nosocomial pneumonia, although colonisation does not always imply infection. However, susceptibility to pneumonia is inversely related to a patient's basic health (Aksamit, 1993; Artenstein and Cross, 1993; Dick *et al.*, 1988; Dropulic *et al.*, 1995). Conditions predisposing to *P. aeruginosa* pneumonia are similar to those mentioned for bacteremia.

Bacteremic pneumonia, with organisms isolated from both the lung and bloodstream, resembles bacteremia and pneumonia in clinical presentation. Prognosis is bleak with this syndrome. Mortality rates

of 80-100% are observed, compared to 27-50% for bacteremia and 30-60% for pneumonia (Aksamit, 1993).

Chronic pulmonary colonisation by *P. aeruginosa* in the lungs of patients with cystic fibrosis results in frequent acute episodes of pneumonia and chronic bronchiectasis, but rarely in bacteremic pneumonia (Aksamit, 1993). Chronic infection leads to the obstruction of the airways, respiratory distress, and eventually death (Gilligan, 1991; Romling *et al.*, 1994).

Otolaryngologic infections due to *P. aeruginosa* range from superficial and self-limiting to life-threatening (Artenstein and Cross, 1993). The most serious ear infection due to this organism is malignant otitis externa, usually resulting from a failure of topical therapy, and resulting in an invasive disease-destroying tissue which may progress to osteomyelitis at the base of the skull and possible cranial nerve abnormalities (Artenstein and Cross, 1993). Other ear infections associated with *P. aeruginosa* include external otitis (swimmer's ear), otitis media, chronic suppurative otitis media, and mastoiditis (Artenstein and Cross, 1993; Legent *et al.*, 1994; Kenna, 1994; Pollack, 1992).

*P. aeruginosa* is the leading cause of gram-negative ocular infections, presenting as keratitis or endophthalmitis (Holland *et al.*, 1993; Chatterjee *et al.*, 1995; Bukanov *et al.*, 1994). Predisposing factors include the use of contact lenses (in particular their cleaning and storage solutions), trauma, burns, ocular irradiation, compromised host defences, and systemic infections (Holland *et al.*, 1993; Pollack, 1992; Imayasu *et al.*, 1994; Stapleton *et al.*, 1995).

Moisture is the paramount defining factor in *P. aeruginosa* growth. Normal dry skin does not support growth, whereas moist skin enables the organism to flourish. For this reason, dermatologic infections with *P. aeruginosa* tend to be more prevalent in moist tropical and subtropical climates (Bodey *et al.*, 1983) or to be associated with the use of swimming pools, hot tubs or whirlpools (Gustafson *et al.*, 1983; Trueb *et al.*, 1994; Vesaluoma *et al.*, 1995). The use of contaminated "loofah" cosmetic sponges is another source of *P. aeruginosa* infection (Bottone and Perez, 1993; 1994; Fisher, 1994). Folliculitis, pyoderma, cellulitis and ecthyma gangrenosum are all dermatologic infections in which *P. aeruginosa* has been implicated (Pollack, 1992; Artenstein and Cross, 1993; Gustafson *et al.*, 1983; Fisher, 1994; Noble, 1993).

*P. aeruginosa* is a frequent isolate from wounds, particularly those contaminated with soil, plant material or water. Its presence may reflect colonisation as opposed to infection, which is a consequence of its ubiquitous distribution in nature (Artenstein and Cross, 1993; Pollack, 1992). Puncture wounds, particularly those penetrating to bone, may result in osteomyelitis or osteochondritis. The former is common in intravenous drug abusers (Artenstein and Cross, 1993) and the latter in puncture wounds to the foot in children and diabetics (Lavery *et al.*, 1994; Pollack, 1992; Jarvis and Skipper, 1994). The wearing of tennis shoes (sneakers) at the time of puncture injury increases the chance of *P. aeruginosa* infection (Pollack, 1992; Lavery *et al.*, 1994; Fisher *et al.*, 1985).

In rare cases, *P. aeruginosa* has been associated with meningitis or brain abscess (Pollack, 1992) and infection of the gastro-intestinal tract (Artestein and Cross, 1993). Both conditions are nosocomially acquired, occurring in patients suffering from malignancies, invasive procedures or neutropenia (Pollack, 1992; Artenstein and Cross, 1993).

### *P. fluorescens*

*P. fluorescens* has occasionally been associated with human infection. The inability of most strains to grow at normal human body temperature (Palleroni, 1992a) restricts invasion and subsequent disease promotion. This organism has the ability to grow at 4°C (Gilardi, 1991). This characteristic, along with the observation that it is isolated from the skin of a small percentage of blood donors, makes it an occasional

contaminant of whole blood and blood products (Puckett *et al.*, 1992; Stenhouse and Milner, 1992). Pseudobacteremia may result from the infusion of contaminated products (Scott *et al.*, 1988; Simor *et al.*, 1985; Gottlieb *et al.*, 1991; Foreman *et al.*, 1991) or from the use of contaminated equipment (Anderson and Davey, 1994).

*P. fluorescens* has been occasionally isolated from patients with AIDS (Franzetti *et al.*, 1992; Roilides *et al.*, 1992), where it caused bacteremia and urinary tract, ocular and soft tissue infections. Chamberland *et al.* (1992), in their across-Canada survey of septicemia, found that 1.5% of isolates were *P. fluorescens*. It is apparent that *P. fluorescens* can be an opportunistic pathogen in cancer patients and in others who are severely immunocompromised, but that it is of little concern to immunocompetent individuals. *P. fluorescens* is occasionally found in sputa of patients with cystic fibrosis, although its role as a pathogenic factor has yet to be resolved.

### *P. fragi*

*P. fragi* is one of the pseudomonads associated with food spoilage (Barrett *et al.*, 1986; Drosinos and Board, 1995; Greer, 1989) and is commonly isolated from milk products, pork and lamb. A search of the literature dating back to 1966 failed to reveal any association between *P. fragi* and human disease.

### *P. putida*

*P. putida* is a rare opportunistic pathogen in immunocompromised individuals. Like *P. fluorescens*, this organism can grow at 4°C in whole blood and blood products and is consequently an occasional source of pseudobacteremia (Pitt, 1990; Taylor *et al.*, 1984; Tabor and Gerety, 1984). Septicaemia and septic arthritis due to *P. putida* in immunocompromised patients have been reported (MacFarlane *et al.*, 1991; Madhavan *et al.*, 1973) and bacteremia in AIDS patients can occur at low frequency (Roilides *et al.*, 1992). All syndromes appear to be associated with breaching of the patient's mechanical defences, either associated with transfusion or following placement of in-dwelling catheters.

### *P. chlororaphis*, *P. syringae*, *P. tolaasii*

A search of the literature dating back to 1966 failed to reveal any association between these species and human disease. The possibility does exist that an incomplete identification has failed to speciate these organisms, and that they are reported in the literature as *Pseudomonas* sp.

## 2. Communicability

*P. aeruginosa*, the species of most concern in the rRNA group I pseudomonads, has a ubiquitous distribution at a low frequency in nature (Romling *et al.*, 1994a). Outside of the hospital environment, 20 to 30% of people harbour faecal *P. aeruginosa*. This frequency increases during hospitalisation as a result of contact with an environment in which the organism is more common. Both healthy individuals and patients with *P. aeruginosa* infections may serve as reservoirs for infection in hospitals.

*P. aeruginosa* is an important cause of nosocomial infections. It is particularly a problem in burn units, neonatal units, and wards housing leukemia and other cancer patients (Bergen, 1981). Nosocomial infections may spread by transmission 1) directly between patients; 2) via medical personnel; 3) via inanimate objects which may serve as reservoirs or vectors; and 4) from the normal flora of the patient (*i.e.* autoinfection).

Most types of hospital equipment or utensils can serve as a source of infection, including pharmaceutical products, disinfectants, water jugs, table tops, trays, urine bottles, urethral catheters, anaesthetic equipment, and respiratory apparatus. Transmission may also occur via food stuffs such as

strawberries, plums and other fruit, vegetables, frozen poultry, refrigerated eggs, lemonade, raw milk, and any equipment or utensil involved in the preparation or serving the food.

### 3. Infective dose

Infective dose for the fluorescent pseudomonads is not really relevant, since infection usually occurs in immunosuppressed individuals. Most patients suffering from cystic fibrosis acquire a *P. aeruginosa* infection at some stage of their lives, resulting in frequent, recurrent bouts of pneumonia. Mortality in such cases may reach 100%.

### 4. Host range, possibility of alteration

*P. aeruginosa* has a broad host range which includes humans, animals, and some plants. It converts from a non-mucoid state to a mucoid, alginate-producing variant in the lungs of CF patients. The mucoid form is almost exclusive to colonisation of this site. Upon *in vitro* propagation, the mucoid strains isolated from CF lungs may undergo a spontaneous reversion to the non-mucoid form (Maharaj *et al.*, 1992).

### 5. Capacity for colonisation

Fluorescent pseudomonads may be found in the normal bacterial flora of the intestines, mouth or skin of humans or animals. Colonisation is harmless under normal circumstances. In immunosuppressed or immunocompromised patients the capacity for colonisation by *P. aeruginosa* is high.

### 6. Possibility of survival outside the human host

rRNA group I fluorescent pseudomonads do not require human or animal hosts for survival. Most are common residents of soil, rhizosphere, sediment, and aquatic habitats. These generally moist environments provide natural reservoirs for the organisms. The pseudomonads have modest nutritional demands and can survive for months in tap water, distilled water, sink drains, or any other moist environment.

### 7. Means of dissemination

The fluorescent pseudomonads are ubiquitous micro-organisms. Anyone (not only infected individuals), or anything, may serve as a source or vector for dissemination (refer also to 10 and 26).

### 8. Biological stability

In *P. aeruginosa* infections of the CF lung, a transition from a non-mucoid to a mucoid, alginate producing variant is observed, indicating the pleomorphic nature of this organism. Furthermore, the level of toxin production varies with the isolate, suggesting that expression levels of chromosomally encoded genes are subject to strain differences. Recent studies indicate that this variation is attributable to the variable position of the genes on the chromosome, due at least in part to chromosome reassortment and the movement of IS-like sequences (Vasil *et al.*, 1990).

### 9. Antibiotic-resistance patterns

#### *P. aeruginosa*

*P. aeruginosa* is naturally resistant to many widely used antibiotics. Resistance in part is thought to be the result of an impermeable outer membrane and the production of extracellular polysaccharides (Quinn, 1992). The organism is usually resistant to low levels of kanamycin, penicillins (with the exception of the anti-pseudomonal penicillins: carbenicillin, ticarcillin, piperacillin), most of the first and second generation

cephalosporins, chloramphenicol, nalidixic acid, tetracyclines, erythromycin, vancomycin, sulfonamide, trimethoprim and clindamycin (Wiedemann and Atkinson, 1991). Antibiotic resistance is often due to the presence of plasmids (Table 4.7). Individual strains may be resistant to antibiotics to which the species is generally susceptible. For this reason, antibiotic resistance patterns should not be relied on for species verification, but should be assessed on a case-by-case basis.

### *P. fluorescens* and *P. putida*

Antibiotic resistance patterns for *P. fluorescens* and *P. putida* are difficult to assess, since only small numbers of isolates have been tested in controlled studies. The organisms tested are susceptible to low levels of kanamycin and resistant to carbenicillin and gentamicin, two of the antibiotics still in use against *P. aeruginosa* (Pitt, 1990). Again the use of antibiotic resistance/susceptibility profiles should be regarded with caution, since variation within a species may be great.

### *P. chlororaphis*, *P. fragi*, *P. syringae*, *P. tolaasii*

Antibiotic susceptibility patterns for these species were not found in the literature searched.

## 10. Toxicogenicity

The pathogenicity of *P. aeruginosa* is accredited to the wide array of virulence-associated factors produced by some if not all strains. Pili act as adhesins to a variety of cell types and enable the organism to colonise epithelial surfaces (Prince, 1992). Once established, the bacteria secrete a number of extracellular products capable of tissue damage and facilitating dissemination of the bacteria (Plotkowski *et al.*, 1994). Proteases (including elastase), exotoxin-A, exoenzyme-S, phospholipase-C, exolipase, rhamnolipid, alginate, cytotoxin, high molecular weight leukocidin and endotoxin have all been implicated in pathogenesis (Fick, 1993; Govan and Nelson, 1992; Holder, 1993; Holland *et al.*, 1993; Jaeger *et al.*, 1991; McCubbin and Fick, 1993; Kudoh *et al.*, 1994; Lutz *et al.*, 1991; Noda *et al.*, 1991).

Exotoxin-A (ETA) and exoenzyme-S are ADP-ribosyltransferases which inhibit protein synthesis in the eukaryotic cell. ETA is produced during the decline of the *P. aeruginosa* growth cycle. Its synthesis is dependent on the iron concentration in the growth medium (Stephen and Pietrowski, 1986). The levels of both ETA and exoenzyme-S vary with the isolate examined.

Phospholipase-C (PLC) is another extracellular enzyme produced by *P. aeruginosa* which is toxic in micro or sub-microgram levels. PLC preferentially degrades phospholipids, which are plentiful in the eukaryote cell. In addition, one of the substrate products of lipid degradation by PLC (diacylglycerol) can have toxic effects on the host animal by inducing the production of potent substances (arachidonic acid metabolites and protein kinase C). These by-products alter eukaryotic cell metabolism and incite inflammatory responses.

Elastase, one of the extracellular proteases, degrades elastin, collagen, human immunoglobulin and serum  $\alpha$ -1-proteinase inhibitor (Iglewski *et al.*, 1990), activities which help evade the immune response and sponsor tissue invasion. Alkaline protease, another of the extracellular proteases, is active on IgA, cytokines (TNF- $\alpha$ ; IFN- $\gamma$ ; IL-2), lactoferrin and transferrin, fibrinogen, and fibrin (Shibuya *et al.*, 1991; Doring *et al.*, 1988; Frick *et al.*, 1985; Parmely *et al.*, 1990). These enzymatic activities promote disruption of respiratory cilia and increased vascular permeability, which probably contribute to establishment in the lung and resulting pneumonia.

The toxigenic potential of other species of *Pseudomonas* is less well studied. Proteases and phospholipases have been detected in some strains of *P. fluorescens* and *P. putida*, but their significance in human infection has yet to be elucidated.

## 11. Allergenicity

Fluorescent pseudomonads have not been described as potent allergens. However, they do possess endotoxin (lipopolysaccharide), which may precipitate an allergic response in some individuals.

**Table 4.7** Examples of plasmids encoding for drug resistances in *P. aeruginosa*

Plasmid	Resistances encoded
RP8	carbenicillin, kanamycin, neomycin, tetracycline
RP1-1	carbenicillin
R9169	carbenicillin, kanamycin, neomycin, tetracycline
R6886	carbenicillin, kanamycin, neomycin, tetracycline
RP8	carbenicillin, kanamycin, neomycin, tetracycline
R2-72	carbenicillin, streptomycin, kanamycin
R38-72	tetracycline, streptomycin
R39-72	tetracycline, streptomycin
R931	tetracycline, streptomycin
R679	streptomycin, sulphonamide
R1162	streptomycin, sulphonamide
R3108	streptomycin, sulphonamide, tetracycline
R209	streptomycin, sulphonamide, gentamicin
R130	streptomycin, sulphonamide, gentamicin
R716	streptomycin
R503	streptomycin
R5265	streptomycin, sulphonamide
R64	ampicillin, carbenicillin, sulphonamide, gentimicin, kanamycin
R40a	ampicillin, anamycin, paromycinin, sulphonamide

Source : taken from Trevors (1991)

## 12. Availability of appropriate prophylaxis and therapies

### *P. aeruginosa*

Antibiotic therapy for *P. aeruginosa* depends upon the site of infection and the relative susceptibility of the particular strain to the antibiotics tested. Generally, the species is susceptible to very few antibiotics. Ceftazidime, cefsulodin, imipenem, ticarcillin-clavulanic acid, azlocillin, piperacillin, the aminoglycosides, colistin and ciprofloxacin are some of the antibiotics with a high percentage of susceptible isolates (Chamberland *et al.*, 1992; Wiedemann and Atkinson, 1991; Legent *et al.*, 1994). Combination therapy using two effective antibiotics may increase the clinical cure rate in some infections (Lucht *et al.*, 1994), and synergistic combinations of an aminoglycoside with a  $\beta$ -lactam (that has activity against *Pseudomonas*) have continued to be effective (Sepkowitz *et al.*, 1994). The particular antibiotic regime selected will depend, however, on the strain in question and cannot be answered in a generic manner.

### *P. fluorescens* and *P. putida*

Ceftazidime (Jones *et al.*, 1989; Watanabe *et al.*, 1988), imipenem (Jones *et al.*, 1989) and meropenem (Jones *et al.*, 1989) have been described as active against *P. fluorescens*. Antibiotics active against *P. putida* are ceftazidime, carbapenems, aminoglycosides, tetracyclines and polymyxin B (Kropec

*et al.*, 1994; Bergen, 1981; Papapetropoulou *et al.*, 1994). Any possible treatment regime should be proposed for the strain in question and not based on generic information for the species.

***P. chlororaphis*, *P. fragi*, *P. syringae*, *P. tolaasii***

No antibiotic susceptibility data for these species were found in the literature searched.

## **V. Environmental and Agricultural Considerations**

### **1. Natural Habitat and Geographic Distribution. Climatic Characteristics of Original Habitats**

#### **A. General considerations**

*Pseudomonas* species have been isolated from a wide variety of habitats including soils, fresh or sea water, clinical specimens and laboratory reagents (including distilled water), food stuffs and wastes, flowers, fruit, vegetables, and diseased and healthy plants and animals. Many species appear to have a global distribution. For example, beneficial colonizers and *Pseudomonas*-incited plant diseases (such as *P. syringae*) are worldwide in distribution and involve representatives of most major groups of common plants.

Although pseudomonads are often considered to be ubiquitous, there are also many reports of niche specialisation. For instance, the number of epiphytic bacteria (such as *P. syringae*) present on the leaves of newly emerged plants is very low, indicating that the soil does not appear to serve as an important source of inoculum (Lindow, 1992) or habitat. As well, many phytopathogenic pseudomonads can only be isolated from the diseased host. For example, *P. syringae* isolates are generally only found in association with live plants or propagative material, and in these niches they appear as virtually homogeneous populations (Schroth *et al.*, 1981). At present, the distribution of these pathogens in the absence of the host is unclear.

#### **B. *P. aeruginosa***

*P. aeruginosa* is widely distributed in soil (Bradbury, 1986) and water (Palleroni, 1984). It is occasionally isolated from both healthy and diseased plants (Bradbury, 1986). Experiments conducted with lettuce and bean under varying conditions of temperature and humidity indicated that *P. aeruginosa* can colonise these plants under conditions of high temperature and humidity (27°C, 80-95% humidity) (Green *et al.*, 1974). The occurrence declined in lettuce and bean when the temperature and humidity were lowered (16°C, 55-75% humidity).

Cho *et al.* (1975) studied the occurrence of *P. aeruginosa* on the foliage and in the soil of potted ornamental plants in order to determine their importance as a disseminating agent in hospital environments. They concluded that although potted plants are potential carriers for introduction of the species to hospital environments, there is no evidence that these plants constitute a primary source of bacteria for hospital infections. Results of a study to determine the prevalence of bacteria in passerines and woodpeckers suggest that *Pseudomonas* spp., including *P. aeruginosa*, are not uncommon in the gut flora of omnivorous and granivorous birds (Brittingham *et al.*, 1988).

#### **C. *P. chlororaphis***

*P. aureofaciens* (*P. chlororaphis*) was one of the most commonly occurring bacteria in soil, and on roots and leaves of both sugarbeet and spring wheat, during the growing season (De Leij *et al.*, 1994). *P. chlororaphis* has also been isolated from water and from dead larvae of cockchafer, a large European beetle (Palleroni, 1984).

#### **D. *P. fluorescens***

*P. fluorescens* is commonly found on plant surfaces, as well as in decaying vegetation, soil and water (Bradbury, 1986). It can be isolated from soil, water, plants, animals, the hospital environment, and human clinical specimens. It is commonly associated with spoilage of foodstuffs such as fish and meat (Gilardi, 1991). The presence of *P. fluorescens* in the rhizosphere of plants has been widely reported. For example, Milus and Rothrock (1993) found *P. fluorescens* to be a very good coloniser of wheat roots, and Lambert *et al.* (1990) found *P. fluorescens* to be one of the most frequently occurring bacteria on root surfaces in young sugar beet plants in Belgium and Spain.

*P. marginalis* (*P. fluorescens*) is ubiquitous in soil and is often an internal resident of plant tissues (Schroth *et al.*, 1992). Cuppels and Kelman (1980) detected *P. marginalis* in a Wisconsin river and lake, field soils, root zones of potato plants, washwater from a potato chip processing plant, and decaying carrot and cabbage heads. Strains were found in Wisconsin soils just after the spring thaw, and thus probably overwintered there.

#### **E. *P. fragi***

*P. fragi* has been found associated with refrigerated meat and dairy products (Jay, 1992).

#### **F. *P. putida***

*P. putida* is very common in soils and plant rhizospheres (Palleroni, 1984). Gilardi (1991) indicated the species can be isolated from soil, water, plants, animal sources, the hospital environment, and human clinical specimens. It can be isolated from soil and water after enrichment in mineral media with various carbon sources.

*P. putida* appears to have a broad global distribution. Sisinthy *et al.* (1989) isolated the species from soil samples collected in, and around, a lake in Antarctica. However, particular strains may have a more restricted distribution. Chanway and Holl (1993) studied strains obtained from spruce seedling rhizospheres at two different locations in British Columbia, Canada, and found two distinct strains based upon analysis of fatty acids. When the origin of the spruce seed was matched with that of the inoculated *P. putida* strain, a significant increase in the amount and rate of seedling emergence was detected compared to unmatched tests of seedling emergence, suggesting ecotype specificity of strains.

#### **G. *P. syringae***

*P. syringae* occurs naturally among the microflora that inhabit the leaf surface of plants that are typically found in temperate and Mediterranean climates (Wilson and Lindow, 1994; Bradbury, 1986). *P. syringae* survives in association with the host plant and propagative material from the host plant. There is little evidence to suggest that these bacteria survive in soil. They may, however, survive in soil in association with residues of diseased plants, having some capacity to colonise root systems (both host and non-host plant). Stone or pome fruit pathogens, such as *P. syringae*, exist in lesions, cankers or tumours. Inoculum is therefore available for dissemination under favourable environmental conditions. Most of the *P. syringae* group appears to have the capacity to survive as epiphytes on protected parts of healthy leaves, in the buds of the host, and even on non-host plants.

#### **H. *P. tolaasii***

*P. tolaasii* is a natural inhabitant of peat and lime used for casing material in the production of commercial mushrooms, and can be easily isolated from compost after pasteurisation (Howard *et al.*, 1994). In the commercial production of mushrooms, high relative humidity and surface wetness encourage

the expression of symptoms of brown blotch caused by *P. tolaasii* (Howard *et al.*, 1994). Symptoms of brown blotch occur more frequently on mushrooms that remain wet for a long time, and in places where they touch one another (Howard *et al.*, 1994). Brown blotch, the mushroom disease caused by *P. tolaasii*, has been reported on all continents except Africa (Bradbury, 1986; Suyama and Fujii, 1993).

## **2. Significant Involvement in Environmental Processes, Including Biogeochemical Cycles and Potential for Production of Toxic Metabolites**

Pseudomonads can have a significant involvement in a variety of environmental processes, including important biogeochemical cycles. For example, certain *Pseudomonas* species have the capacity for denitrification producing dinitrogen gas from nitrate. These species include *P. aeruginosa*, *P. fluorescens* (biotypes I and III) and *P. chlororaphis* (Palleroni, 1984).

### ***Toxic metabolites of hazardous wastes***

The microbial degradation of a hazardous waste may result in mineralisation of the parent waste, or in partial degradation of the parent waste to products which may be toxic. Some micro-organisms may not initially produce problematic metabolites. However, loss of a lower portion of a degradative pathway due to genetic instability may result in the generation of toxic metabolites. These toxic metabolites may result in death of the cell, thus limiting the metabolite's production. The metabolite may also be released from the cell to soil or water and become rapidly inactivated or mineralised by other physical or biological processes. On the other hand, some metabolites may be released from the cell, remain stable in the environment, and have toxic effects equivalent to, or greater than, the parent hazardous waste. There is also a possibility that a micro-organism will not produce a metabolite of concern when presented with a single waste, but will produce toxic metabolites in the presence of a complex mixture of related compounds.

Many examples of hazardous waste metabolites have been detected in laboratory experiments, but no well-documented field studies on metabolite formation have been conducted. In many instances, the metabolites produced by one organism will be degraded further or mineralised by others in the immediate environment. For example, TCE epoxide and phosgene are likely degradation products from methanotrophic degradation of trichloroethylene and chloroform, respectively (Alvarez-Cohen and McCarty, 1991). Although these compounds are toxic in mammalian systems, both are also highly reactive and would likely react intracellularly and/or not persist in the environment once released from the cells. Examples of the potential for hazardous metabolites include the production and accumulation of formamide from cyanide as a result of cyanide degradation by *P. fluorescens* strain NCIMB 11764 (Kunz, *et al.*, 1992). As well, Castro and Belser (1990) demonstrated that *P. putida* PpG-786 can dehalogenate 1,1,2-trichloroethane by two pathways under aerobic conditions. The dominant pathway is oxidative and leads to chloroacetic acid and glyoxylic acid. However, a competitive reductive pathway occurs simultaneously and yields vinyl chloride exclusively.

Complex mixtures can result in dead-end metabolite production, or failure to degrade one of the parent compounds, even though the individual wastes can be mineralised individually. Benzene, toluene, and *p*-xylene (BTX) are common contaminants of drinking water, and each individual BTX compound can be mineralised by naturally occurring organisms. However, a combination of the three cannot be mineralised naturally, and can result in accumulation of 3,6-dimethylcatechol from *p*-xylene and a lack of degradation of benzene (Lee *et al.*, 1995).

### 3. Pathogenicity – Host Range, Infectivity, Toxigenicity, Virulence, Vectors

#### A. General considerations

The fluorescent rRNA group I pseudomonads exhibit a range of pathogenicity characteristics. Some species have not been implicated in animal or plant disease. Other species may be opportunistic pathogens for weakened individuals. The fluorescent rRNA group I pseudomonads also include plant pathogens.

#### B. *P. aeruginosa*

##### *Pathogenicity to animals*

*P. aeruginosa* may be found as part of the normal bacterial flora of the intestines, mouth or skin of animals (e.g. cattle, dogs, horses, pigs). It has a broad host range among animals, which may also extend to plants. Under normal circumstances, colonisation is harmless and infection only occurs when local or general defence mechanisms are reduced. *P. aeruginosa* is usually associated with disease in individuals with low resistance to infection.

In susceptible hosts *P. aeruginosa* may cause infection at any site, particularly wounds and the respiratory tract. It can cause endocarditis, meningitis, pneumonia, otitis, vaginitis and conjunctivitis. Host defence mechanisms against *P. aeruginosa* are very low in mink and chinchilla, in which the bacterium can spread rapidly, causing fatal disease (Bergen, 1981).

*P. aeruginosa* has been associated with disease in pigs, sheep and horses (Hungerford, 1990), as well as cattle (Hamdy *et al.*, 1974). Sheep inoculated epicutaneously with *P. aeruginosa*, and then wetted, can rapidly develop a bacterial exudative dermatitis (Hungerford, 1990). *P. aeruginosa* has been reported as the etiological agent in outbreaks of acute infectious disease in mink (Wang, 1987) and was the suspected etiological agent in a report of fatal bronchopneumonia and dermatitis in an Atlantic bottle-nosed dolphin (Diamond and Cadwell, 1979). It has also been reported to be associated with pathogenicity in ducks (Safwat *et al.*, 1986), turkeys (Hafez *et al.*, 1987), Japanese ptarmigan (Sato *et al.*, 1986), and pheasant chicks (Honich, 1972) and to be the causal agent of a disease in broiler fowl in several countries.

*P. aeruginosa* was reported as one of the causative agents of infectious stomatitis or “mouthrot” in snakes (Draper *et al.*, 1981), although it has been suggested that it is an opportunistic invader rather than an exogenous pathogen in snakes (Draper *et al.*, 1981; Jacobson *et al.*, 1981). Frogs (*Rana pipiens*) that were intraperitoneally injected with high doses ( $10^4$ - $10^6$  bacteria) of *P. aeruginosa* showed significant mortality under stressful conditions (Brodtkin *et al.*, 1992). *P. aeruginosa* has been associated with pathogenicity in Nile fish (Youssef *et al.*, 1990) and catfish (*Clarias batrachus*) (Manohar *et al.*, 1976) and as the etiological agent of fin rot in *Rhamdia sapo* (Angelini and Seigneur, 1988).

*P. aeruginosa* has been associated with pathogenicity in the tobacco hornworm (*Manduca sexta*) (Horohov and Dunn, 1984) and seven species of Lepidoptera, including the silkworms *Pericallia ricini* and *Bombyx mori* (Som *et al.*, 1980). Experimental inoculation of honeybees, by dipping in a bacterial suspension of *P. aeruginosa*, resulted in a 70% death rate within 50 hours (Papadopoulos-Karabela *et al.*, 1992). Dorn (1976) reported *P. aeruginosa* to be responsible for disease outbreaks in laboratory populations of the milkweed bug *Oncopeltus fasciatus*.

The abundant extracellular products of *P. aeruginosa* are thought to contribute to its adverse effects. These products include toxin A, alkaline protease, alkaline phosphatase, lipase, phospholipases and elastase. Toxin A is toxic to animals, with a mean lethal dose in mice of about 0.2 µg when injected intraperitoneally or 0.06 µg when injected intravenously (Nicas and Iglewski, 1986). Toxin A is produced

by about 90% of clinical isolates, and a chromosomal location has been established for the structural gene (Nicas and Iglewski, 1986). Most strains produce several extracellular proteases. For mice injected intravenously, the LD<sub>50</sub> of the alkaline protease and the elastase is 375 and 300 µg respectively (Nicas and Iglewski, 1986). *P. aeruginosa* proteases are reported to be toxic to insects (*Galleria mellonella*) (Lysenko, 1974). *P. aeruginosa* also produces the haemolytic extracellular product phospholipase C, which causes hepatic necrosis and pulmonary edema when injected interperitoneally, and rhamnolipid, which has an LD<sub>50</sub> of 5 mg when injected interperitoneally into mice (Nicas and Iglewski, 1986).

#### ***Pathogenicity to plants***

*P. aeruginosa* has been described as an opportunistic invader of plants (Bradbury, 1986). It has been reported to cause blight disease in bean plants (El Said *et al.*, 1982), and to have caused a lethal palm blight (Bradbury, 1986). Slow soft rot has been produced in plant tissue upon inoculation with strains of *P. aeruginosa* isolated from both animals and plants, and lesions and some necrosis have been found in tobacco leaves when inoculated with the bacterium (Bradbury, 1986). In a study involving 46 strains of *P. aeruginosa* isolated from human, plant and soil sources, the ratio of pathogenic to non-pathogenic strains for vegetables was 5:1 (Lebeda *et al.*, 1984).

More recently, two strains of *P. aeruginosa* (a clinical isolate and a plant isolate) have been found to elicit severe soft rot symptoms in the leaves of inoculated *Arabidopsis thaliana* plants from certain ecotypes but not others (Rahme *et al.*, 1995). These authors suggested that a strain that exhibited ecotype specificity would most likely be a true plant pathogen, in contrast to a strain that has no capacity to be a plant pathogen under natural settings but infects plants as a consequence of the artificial environment of a laboratory. The same two strains of *P. aeruginosa* were found to cause significant mortality in a mouse burn model. The authors identified genes encoding three virulence factors (*toxA*, *plcS* and *gacA*) that were required for the full expression of pathogenicity in both plants and animals.

#### ***C. P. chlororaphis***

##### ***Pathogenicity to animals***

A strain of *P. chlororaphis* has been reported to cause disease in salmon fry (*Oncorhynchus rhodurus*) and to kill trout, carp and eel, when inoculated (Egusa, 1992). This strain was judged to be pathogenic to fish (Hatai *et al.*, 1975). *P. chlororaphis* has also been reported to inhibit egg hatch of the nematode, *Criconebella xenoplax*, at a concentration of 2x10<sup>8</sup> cfu/ml (Westcott and Kluepfel, 1993). Shahata *et al.* (1988) reported that *P. chlororaphis* infected chickens.

##### ***Pathogenicity to plants***

*P. chlororaphis* has been reported as the causal agent for a disease in straw mushrooms (*Volvariella volvacea*) in Puerto Rico, characterised by basal soft rot, internal water-soaking and discoloration (Hepperly and Ramos-Davila, 1986).

#### ***D. P. fluorescens***

##### ***Pathogenicity to animals***

*P. fluorescens* can infect a wide range of animals including horses (Sarasola *et al.*, 1992), chickens (Lin *et al.*, 1993), marine turtles (Glazebrook and Campbell, 1990), and many fish and invertebrate species. However, because it is unable to grow at elevated temperatures like that of the human body (Palleroni, 1992a), it is unlikely to be more than a rare opportunistic pathogen for warm-blooded animals.

*P. fluorescens* is considered a secondary invader of damaged fish tissues, but may also be a primary pathogen (Roberts and Horne, 1978; Stoskopf, 1993). The species causes bacterial tail rot and can affect freshwater and saltwater fishes throughout the world (Stoskopf, 1993). *P. fluorescens* releases extracellular proteases upon invasion of the fish host (Li and Flemming, 1968), and morbidity can be quite high. Outbreaks of bacterial tail rot have been reported in goldfish, silver carp, bighead, tench, grass carp, black carp, golden shiner, rainbow trout, European eels, paradise fish, and other labyrinth fishes. Egusa (1992) reviewed *Pseudomonas* diseases in fish and indicated that, in the United States, the disease termed *Pseudomonas* septicemia, due to infection by bacteria related to *P. fluorescens* (AFS-FHS, 1975), is seen in comparatively large numbers in warm-water fish.

Adverse effects associated with *P. fluorescens* in fish species often appear to be linked to stress from transportation or cultivation of fish. For example, *P. fluorescens* has been associated with disease in the cultivation of rainbow trout, *Oncorhynchus mykiss* (Barros *et al.*, 1986), Atlantic salmon, *Salmo salar* (Carson and Schmidtkne, 1993), chinook salmon, *Oncorhynchus tshawytscha* (Newbound *et al.*, 1993), sea bream, *Eyynniss japonica* (Kusuda *et al.*, 1974), bighead carp, *Aristichthys nobilis*, and silver carp, *Hypophthalmichthys molitrix* (Petrinec *et al.*, 1985), catfish and carp (Gatti and Nigelli, 1984), tench (Ahne *et al.*, 1982), and tilapia species (Okaeme, 1989; Miyashita, 1984; Miyazaki *et al.*, 1984).

Barker *et al.* (1991) found that exposure of high numbers of *P. fluorescens* to egg surfaces of rainbow trout (*Oncorhynchus mykiss*) during the initial stages of incubation poses a threat to egg survival. Conversely, *P. fluorescens* was not pathogenic when injected into brown trout (Smith and Davey, 1993) or silver mullet fish (*Mugil curema*) (Alvarez and Conroy, 1987).

*P. fluorescens* has also been implicated in pathogenicity to some invertebrates. James and Lighthart (1992) determined an  $LC_{50}$  for the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> instar larvae of a coleopteran insect (*Hippodamia convergens*) ( $4.8 \times 10^9$ ,  $2.8 \times 10^{10}$ ,  $3.9 \times 10^9$ , and  $3.2 \times 10^{11}$  CFU/ml, respectively) and concluded that *P. fluorescens* is a weak bacterial pathogen. *P. fluorescens* has also been reported to be associated with pathogenicity in the mosquitoes *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* (Murty *et al.*, 1994) and in the field slug *Deroeras reticulatum* (Wilson *et al.*, 1994). However, Gentner *et al.* (1993) studied the effects of *P. fluorescens* on eastern oysters (*Crassostrea virginica*) and found no signs of infectivity or pathogenicity.

### **Pathogenicity to plants**

*P. fluorescens* is generally considered to be a saprophyte rather than a plant pathogen (Bradbury, 1986), although Ormrod and Jarvis (1994) considered it to be an opportunistic pathogen causing soft rot in plants. *P. fluorescens* biovar 2 (*P. marginalis*), however, is actively pectinolytic, causing soft rot of various plants, and is considered a plant pathogen (Tsuchiya *et al.*, 1980; Hildebrand, 1989; Membre and Burlot, 1994; Brock *et al.*, 1994). Bradbury (1986) recognised three pathovars in *P. marginalis* which cause soft rot in a wide range of vegetables and other plants.

A number of studies have reported adverse effects associated with *P. fluorescens* and plants (Gaudet *et al.*, 1980; Anson, 1982; Hwang *et al.*, 1989; Richardson, 1993; Ozaktan and Bora, 1994). Tranel *et al.* (1993) found that *P. fluorescens* strain D7 inhibited root growth of downy brome (*Bromus tectorum*) by production of a phytotoxin. Sellwood *et al.* (1981) confirmed pathogenicity experimentally for an atypical *P. fluorescens* biotype I on chicory plants and suggested that the group *P. fluorescens* does not solely comprise saprophytes. However, other studies have found no adverse effects on plants from inoculations with *P. fluorescens* (Arsenijevic, 1986; Arsenijevic and Balaz, 1986; Surico and Scala, 1992). At present, the epidemiology of pathogenic strains of *P. fluorescens* is not well understood (Hildebrand, 1994).

Liao (1991) has cautioned that, at present, it is not known for sure that *P. fluorescens* strains are non-pathogens and do not cause deleterious effects on plants. Liao indicated that the *pel* gene encoding production of pectate lyase (an enzyme which contributes ability to cause soft rot in plants) is well conserved in fluorescent pseudomonads, and may exist and remain repressed in certain strains or species which exhibit non-pectolytic phenotypes under laboratory conditions. Liao further indicated that saprophytic strains of *P. fluorescens* can be induced to become pathogenic and raised a concern about the safety of using the species as a biocontrol agent.

#### **E. *P. fragi***

##### ***Pathogenicity to animals***

No reports were found of *P. fragi* as an animal pathogen.

##### ***Pathogenicity to plants***

No reports were found of *P. fragi* as a plant pathogen.

#### **F. *P. putida***

##### ***Pathogenicity to animals***

*P. putida* can infect a variety of animals including goats (Hungerford, 1990), koala (Ladds *et al.*, 1990), turkey (Ononiwu, 1980) and fish (Kusuda and Toyoshima, 1976). However, because it is unable to grow at elevated temperatures like that of the human body (Palleroni, 1992a), it is unlikely to be more than a rare opportunistic pathogen for warm-blooded animals.

Kusuda and Toyoshima (1976) reported *P. putida* to be a pathogen to cultivated yellowtail fish. However, there have been no reports on the epizootiology, symptoms, or histological or pathological findings, and the disease has not been well-defined (Egusa, 1992). Austin and McIntosh (1991) considered *P. putida* to be one of a variety of gram-negative bacteria pathogens of potential concern to farmed and wild fish. *P. putida* has also been associated with pathogenicity in the snail, *Biomphalaria glabrata* (Cheng, 1986), the crayfish (Boemare and Vey, 1977), and the olive fly (Haniotakis and Avtizis, 1977).

##### ***Pathogenicity to plants***

*P. putida* was included in the *Guide to Plant Pathogenic Bacteria* solely because its multiplication in the rhizosphere of paddy rice plants has been implicated in "suffocation disease", which arises under conditions of poor drainage (Bradbury, 1986). Studies have reported that *P. putida* is not pathogenic to mushrooms (Ozakatan and Bora, 1994) or crucifer plants (Shaw and Kado, 1988).

Liao (1991) has cautioned that, at present, it is not known for sure that *P. putida* strains are non-pathogens and do not cause deleterious effects on plants. Liao indicated the *pel* gene encoding production of pectate lyase (an enzyme which contributes ability to cause soft rots in plants) is well conserved in fluorescent pseudomonads and may exist and remain repressed in certain strains or species which exhibit non-pectolytic phenotypes under laboratory conditions. Homologous sequences were found in strains of *P. putida*, and Liao raised a concern about the safety of using *P. putida* as a biocontrol agent.

### G. *P. syringae*

#### *Pathogenicity to animals*

No reports were found of *P. syringae* as an animal pathogen.

#### *Pathogenicity to plants*

*P. syringae* is principally an assemblage of foliar pathogens, although it occurs as both pathogenic and epiphytic (non-pathogenic) strains. The species has a broad range of potential plant hosts (Table 4.8). Pathogenic strains can exhibit both pathogenic (*i.e.* disease-causing) and epiphytic behaviours on susceptible hosts (Crosse, 1959). The initiation of infection results when a threshold level of bacteria is reached on the leaf surface; in the case of *P. syringae* pv. *syringae* this is reported to be  $10^4$  cfu gm<sup>-1</sup> tissue (Hirano and Upper, 1983).

The association between rain and the onset of foliar blights caused by *P. syringae* is well recognised. Rain appears to stimulate the differential growth of pathogenic *P. syringae* isolates from the heterogeneous populations (pathogenic and non-pathogenic strains). Rain-triggered growth of *P. syringae* results in the establishment of large pathogenic populations required for disease development (Hirano and Upper, 1992).

There appears to be a distinctive set of symptoms associated with each causal agent. *Pseudomonas syringae* pv. *savastoni* incites tumourous outgrowths on stems and leaves of oleander and olive under natural conditions. These symptoms have been found to be associated with the production of the auxin, indole acetic acid (IAA), in tissues infected with the bacterium. Furthermore, chlorosis, a common symptom when plants are infected by a number of pathogens belonging to the *P. syringae* group, is indicative of production of a toxin. For example, halo blight of beans caused by *P. syringae* pv. *phaseolicola* is mediated by the toxin, phaseolotoxin. Other phytopathogenic pseudomonads producing toxins are illustrated in Table 4.9.

The *Dictionary of Natural Products* (Chapman and Hall, 1995) lists the following toxins produced by various strains of *P. syringae*: 1H-Indole-3-carboxaldehyde, octicidin (phytotoxin), phascolotoxin (phytotoxin), N-Phosphosulfamylornithine (phytotoxin), syringomycin (phytotoxin), syringostatin A (phytotoxin), syringostatin B (phytotoxin), syringotoxin B (phytotoxin), tagetitoxin (phytotoxin), coronafacic acid (induces chlorosis in plants), halotoxin (phytotoxin), tabtoxin (phytotoxin). Coronatine (phytotoxin) is also produced by certain strains of *P. syringae* (Cuppels and Ainsworth, 1995). Gross (1985) determined that syringomycin production was stimulated by iron and suppressed by inorganic phosphate, that production occurred between 15 and 27°C, and that a slow growth rate of *P. syringae* favours toxin production.

Table 4.8 Range of plant species susceptible to infection with *P. syringae*

Plant (genus, species)	<i>P. syringae</i> pathovar
<i>Acer</i> spp.	aceris
<i>Aesculus indica</i>	aesculi
<i>Antirrhinum majus</i>	antirrhini
<i>Apium graveolens</i>	apii
<i>Beta</i> spp., <i>Heleanthus annuus</i> , <i>Tropaeolum majus</i>	aptata
<i>Avena sativa</i> , <i>Hordeum vulgare</i> , <i>Triticum aestivum</i>	atrofaciens
<i>Agropyron</i> spp., <i>Agrostis</i> spp., <i>Bromus</i> spp., <i>Dlymus</i> spp., <i>Festuca</i> spp., <i>Lolium</i> spp., <i>Phleum pratense</i> , <i>Stipa</i> spp.	atropurpurea
<i>Corylus avellana</i>	avellanae
<i>Berberis</i> spp.	bereridis
<i>Cannabis sativa</i>	cannabina
<i>Ceratonia siliqua</i>	ciccaronei
<i>Avena</i> spp., <i>Arrhenatherum elatius</i> , <i>Calamagrostis montanensis</i> , <i>Deschampsia caespitosa</i> , <i>Koeleria cristata</i> , <i>Phelum partense</i> , <i>Triticum X Secale</i> , <i>Trisetum spicatum</i> , <i>Zea mays</i>	coronafaciens
<i>Delphinium</i> spp.	delphini
<i>Dysoxylum spectabile</i>	dysoxyl
<i>Eriobotrya japonica</i>	eribotryae
<i>Ficus palmata</i>	fici
<i>Coffea arabica</i>	garcae
<i>Glycine max</i>	glycinea
<i>Helianthus</i> spp.	helianthi
<i>Hordeum vulgare</i> , <i>Triticum aestivum</i>	japonica
<i>Citrullum lanatus</i> , <i>Cucumis</i> spp., <i>Cucurbita</i> spp.	lachrymans
<i>Zea mays</i> , <i>Sorghum bicolor</i>	lapsa
<i>Brassica</i> spp., <i>Raphus sativus</i>	maculicola
Plant (genus, species)	<i>P. syringae</i> pathovar
<i>Nicotiana tabacum</i>	mellea
<i>Morus</i> spp.	mori
<i>Prunus</i> spp.	morsprunorum
<i>Myrica rubra</i>	myricae
<i>Oryza sativa</i>	oryzae
<i>Panicum miliaceum</i>	panici
<i>Malus pumila</i> , <i>Pyrus communis</i>	papulans
<i>Passiflora edulis</i>	passiflorae
<i>Prunus persica</i>	persicae
<i>Phaseolus</i> spp., <i>Pisum sativum</i> , <i>Pueraria lobata</i>	phaseolicola
<i>Philadelphus coronarium</i>	philadelphi
<i>Photinia glabra</i>	photinia
<i>Lathrus</i> spp., <i>Pisum</i> spp., <i>Vicia</i> spp.	pisi
<i>Allium porrum</i>	porri
<i>Primula</i> spp.	primulae
<i>Protea cynaroides</i>	proteae
<i>Ribes aureum</i>	ribicola
<i>Forsythia intermedia</i> , <i>Fraxinus</i> spp., <i>Ligustrum</i> spp., <i>Nerium oleander</i> , <i>Olea</i> spp., <i>Nicotiana tabacum</i>	savastanoi
<i>Sesamum indicum</i>	sesami
<i>Avena sativa</i> , <i>Triticum X Secale</i>	striafaciens
many hosts	syringae
<i>Glycine max</i> , <i>Nicotiana tabacum</i>	tabaci
<i>Ambrosia artemisiifolia</i> , <i>Helianthus</i> spp., <i>Tagetes</i> spp.	tagetis
<i>Camellia sinensis</i>	theae
<i>Capsicum anuum</i> , <i>Lycopersicon esculentum</i>	tomato
<i>Ulmus</i> spp.	ulmi
<i>Viburnum</i> spp.	viburni
<i>Pseudostuga menziesii</i>	

Source : adapted from Bradbury (1986) and Chanway and Holli (1992)

**Table 4.9** Some toxins produced by phytopathogenic *Pseudomonas* spp.

<b>Pseudomonad</b>	<b>Toxin(s)</b>	<b>Mechanism or site of action</b>	<b>Host plant(s)</b>
<i>P. syringae</i>	coronatine		Italian rye grass
pv. <i>atropurpurea</i>	tabtoxin- $\beta$ -lactam	glutamine synthetase	oat
pv. <i>coronafaciens</i>	tabtoxin- $\beta$ -lactam	glutamine synthetase	coffee
pv. <i>garcae</i>	coronatine/ polysaccharide		soybean
pv. <i>glycinea</i>	extracellular polysaccharides		cucumber
pv. <i>lachrymans</i>	coronatine		crucifers
pv. <i>maculicola</i>	coronatine		sour cherry
pv. <i>morsprunorum</i>	phaseolotoxin	ornithine transcarbamoylase	bean, kudzu
pv. <i>phaseolicola</i>	IAA & cytokinins	plant growth regulators	olive, oleander
pv. <i>savastoni</i>	syringomycins	plasma membrane	peach, maize
pv. <i>syringae</i>	syringopeptins		
	syringotoxins		
pv. <i>tabaci</i>	tabtoxin- $\beta$ -lactam	glutamine synthase	tobacco
pv. <i>tagetis</i>	tagetitoxin	chloroplasmic RNA polymerase	marigold
pv. <i>tomato</i>	coronatine		tomato
<i>P. tolaasii</i>	tolaasin	plasma membrane	mushroom

Source : taken from Durbin (1996)

## **H. *P. tolaasii***

### ***Pathogenicity to animals***

No reports were found of *P. tolaasii* as an animal pathogen.

### ***Pathogenicity to plants***

*P. tolaasii* causes Brown blotch (bacterial blotch), the most common bacterial disease of the commercial button mushroom, *Agaricus bisporus* (Howard *et al.*, 1994). This disease can result in serious economic losses. *P. tolaasii* has also been found to cause disease in the oyster mushroom, *Pleurotus ostreatus*, and the shiitake mushroom, *Lentinus edodes* (Suyama and Fujii, 1993).

*P. tolaasii* produces a haemolytic lipodepsipeptide toxin, tolaasin, which is a potent biosurfactant (Hutchison and Johnstone, 1993) and the primary disease determinant of its pathogenicity to the cultivated mushroom, *Agaricus bisporus* (Rainey *et al.*, 1993). Tolaasin is phytotoxic when infiltrated into tobacco leaves (Rainey *et al.*, 1991). Synthesis of tolaasin is chromosomally determined, and it is known that at least five genetic loci are required for tolaasin synthesis (Rainey *et al.*, 1993).

#### 4. Interactions with and Effects on Other Organisms in the Environment

##### A. *P. aeruginosa*

Certain *P. aeruginosa* strains are antagonistic to plant pathogens such as damping-off fungi (Bradbury, 1986; Buysens *et al.*, 1994). Duffy and Defago (1995) found that clinical and plant isolates of *P. aeruginosa* suppressed root diseases of cucumber, maize and wheat caused by soilborne fungi *Gaeumannomyces graminis* var. *tritici*, *Phomopsis sclerotoides*, *Pythium ultimum* and *Rhizoctonia solani*. A soil isolate of *P. aeruginosa* suppressed foliar disease on wheat caused by *Septoria tritici* (Flaishman *et al.*, 1990).

*P. aeruginosa* can have a synergistic effect on the survival of salmonellae, enabling them to survive more than 140 days in double-distilled water (Warburton *et al.*, 1994). It has also been suggested that *P. aeruginosa* may act synergistically with pectolytic bacteria that colonise vegetables, such as *P. marginalis* (*P. fluorescens*) and *Erwinia cartovora* (Bradbury, 1986). A protective immunity against *P. aeruginosa* infection has been reported in mice vaccinated with heat-killed *Lactobacillus casei* (Miake *et al.*, 1985).

*P. aeruginosa* is known to produce 1-phenazinecarboxamide (the amide of 1-phenazinecarboxylic acid), which is active against some phytopathogenic fungi and *Candida albicans*. A related compound, 1-phenazinol, which is active against gram-positive bacteria and fungi, and which shows some viral activity, is also produced by *P. aeruginosa*. 1-phenazinol has an LD<sub>50</sub> of 500 mg/kg in mice dosed intraperitoneally. Pyoluteorin and its 3'-nitro derivative are produced by *P. aeruginosa*. Both compounds have antibacterial, antifungal and herbicidal properties. The LD<sub>50</sub> of the pyoluteorin to mice is 125 mg/kg (Chapman and Hall, 1995). The antibiotic, 2-heptyl-4-hydroxyquinoline N-oxide, is a metabolite of *P. aeruginosa* and is a potent 5'-lipoxigenase inhibitor, with an LD<sub>50</sub> of 40 mg/kg in mice dosed intraperitoneally (Chapman and Hall, 1995).

##### B. *P. chlororaphis*

*P. chlororaphis* has been widely investigated for its ability to enhance plant growth through suppression of deleterious root-colonising bacteria. Compounds known as siderophores are produced by *P. chlororaphis*. These compounds chelate iron, thereby depriving certain root-colonising plant pathogens of iron necessary for their growth (Smirnov *et al.*, 1991).

Many studies have indicated that *P. chlororaphis* has the ability to suppress plant disease. For example, *P. aureofaciens* has been investigated as a biocontrol agent to suppress take-all, the wheat root fungal disease. The ability of *P. aureofaciens* to inhibit *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all, was demonstrated *in vitro* and *in vivo* (Harrison *et al.*, 1993). It is believed that disease suppression is largely due to the production of phenazine antibiotics (Thomashow and Pierson, 1991). Carruthers *et al.* (1995) tested the ability of *P. aureofaciens* to suppress root rot of *Asparagus officinalis* caused by *Phytophthora megasperma* var. *sojae*. *P. aureofaciens* significantly reduced the level of infection and disease severity. Other tests suggested that *P. aureofaciens* had a direct growth stimulatory effect on asparagus, independent of antibiotic production (Carruthers *et al.*, 1995). Berg and Ballin (1994) found *P. chlororaphis* inhibited the growth of the phytopathogenic fungus *Verticillium dahliae*.

When Douglas fir seed was inoculated with *P. aureofaciens*, and grown in pasteurised soil, shoot biomass increased significantly when compared with non-inoculated controls (Chanway and Holl, 1992). *P. aureofaciens* has been found to inhibit mycelial growth of *Rhizoctonia solani* in dual culture between 15 and 30°C (Lee *et al.*, 1990). Inoculation of rice seeds was found to control rice sheath blight in the early growth stages, and seedling blight caused by *R. solani*, *Fusarium moniliforme* and *Pythium ultimum* was suppressed by seed treatment and soil incorporation of *P. aureofaciens* (Lee *et al.*, 1990). In another

experiment, the emergence of sweet corn seedlings from soil infested with *Pythium ultimum* was greatly enhanced by coating the seed with *P. aureofaciens* (Mathre *et al.*, 1994). *P. aureofaciens* has also been evaluated for its ability to suppress *Pythium ultimum* damping off of cucumber seedlings (Sugimoto *et al.*, 1990).

*P. aureofaciens* was antagonistic to *Clavibacter michiganensis* subsp. *sepedonicus*, the bacteria implicated in potato ring rot in greenhouse trials with potato seedlings (de la Cruz *et al.*, 1992). *P. aureofaciens* significantly reduced populations of, and infection by, the ring rot bacteria (de la Cruz *et al.*, 1992). Fukui *et al.* (1994) investigated the relationship between pericarp colonisation by *Pythium ultimum* in sugar beets and the growth of pseudomonads in the spermosphere. They found a positive correlation between the incidence of pericarp colonisation by *Pythium ultimum* and the length of the lag phase of the strain used to inoculate the seeds. England *et al.* (1993) investigated the nodulation of whitebean (*Phaseolus vulgaris* L.) by *Rhizobium phaseoli* in the presence of *P. aureofaciens*. No significant difference was found in the numbers of nodules produced in the presence of *P. aureofaciens* as a result of the symbiotic relationship between *Rhizobium phaseoli* and whitebean roots in vermiculite.

*P. chlororaphis* was observed to interfere with the growth of shiitake mushrooms in field experiments with shiitake cultivated logs (Raaska and Mattila-Sandholm, 1991). Siderophores were produced, however the addition of iron to *in vitro* cultures did not entirely neutralize the growth inhibition of mycelia by *P. chlororaphis*. It was concluded that although iron-binding plays an important role, it is not the only factor involved in the inhibition of shiitake by *P. chlororaphis* (Raaska and Mattila-Sandholm, 1991). A siderophore extracted from *P. aureofaciens* was found to inhibit uptake of ferric iron by maize and pea, and the synthesis of chlorophyll in these plants was reduced (Becker *et al.*, 1985).

*P. aureofaciens* is reported to produce an antibiotic-like compound in iron-rich conditions that inhibits the growth of the plant fungal pathogen *Aphanomyces euteiches* (Carruthers *et al.*, 1994). Mazzola *et al.* (1992) suggested that the production of phenazine antibiotics contributes to the ecological competence of *P. aureofaciens*, and that reduced survival of strains unable to produce the antibiotics is due to diminished ability to compete with the resident microflora. Thomashow *et al.* (1990) found that suppression of take-all is related directly to the presence of the antibiotic phenazine-1-carboxylic acid in the rhizosphere of wheat. In another experiment, 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine were also found to be responsible for take-all suppression in wheat (Pierson and Thomashow, 1992). Pyrrolnitrin [3-chloro-4-(3-chloro-2-nitrophenyl)-1H-pyrrole] is an antifungal compound produced by *P. chlororaphis*; its LD50 in mice dosed orally is 1 g/kg (Chapman and Hall, 1995). The antifungal compound, 1,3,6-trihydroxy-2,4-diacetophenone, has also been isolated from culture media (Harrison *et al.*, 1993).

### ***C. P. fluorescens***

*P. fluorescens* has been recognised as beneficial to plant growth (Weller and Cook, 1986; Kloepper *et al.*, 1988). It can enhance plant growth through production of siderophores, which efficiently complex environmental iron, making it unavailable to other components of the soil microflora. Increased plant yields achieved through the inoculation of plant roots have been mimicked by the application of the siderophore, pseudobactin, isolated from *P. fluorescens*. Antibiotic production by *P. fluorescens* has been recognised as an important factor in its ability to suppress phytopathogens. *P. fluorescens* has also been found to significantly promote nodulation, growth and nitrogen accumulation in faba beans (*Vicia faba*) (Omar and Abd-Alla, 1994). Heat-killed cells had no effect.

Certain strains of *P. fluorescens* can promote the formation of ice crystals in water at temperatures near 0°C (Lindow and Panopoulos, 1988; Lindow, 1992). Large populations of these ice<sup>+</sup> bacteria on plant surfaces can cause frost injury. Only 0.01 to 40% of the total bacteria on plant surfaces are sufficient to cause frost injury. In the absence of these bacteria, water on plants can cool to -40°C.

Smith and Davey (1993) found that *P. fluorescens* strains were able to inhibit *Aeromonas salmonicida* that was isolated from Atlantic salmon with furunculosis. Pre-smolts asymptotically infected with *A. salmonicida* and bathed in a solution containing *P. fluorescens* strains were less likely to develop stress-induced furunculosis than non-treated fish. It was concluded that *P. fluorescens* inhibits *A. salmonicida* by competing for free iron, and that it protects against stress-induced furunculosis by inhibiting *A. salmonicida* on external locations. Kimura *et al.* (1990) found that a strain of *P. fluorescens* biovar I (46NW-04) isolated from the aquatic environment produced an antiviral substance that was effective against fish viruses.

#### D. *P. fragi*

Monitoring of microbial flora succession on minced lamb meat revealed that *P. fragi* was the dominant climax species (Drosinos and Board, 1995). Another study indicated that *P. fragi* dominated the flora on lamb carcasses at both 7 and 30°C (Prieto *et al.*, 1992).

#### E. *P. putida*

*P. putida* is very common in soils and plant rhizospheres, where it seems to have a stimulating effect on plant growth (Palleroni, 1984). *P. putida* has been shown to suppress a variety of plant pathogens and to reduce the incidence of plant disease (Liao, 1989; Gamliel and Katan, 1993; Duijff *et al.*, 1994; Freitas *et al.*, 1991; Defago and Hass, 1990). This may be due in part to its inhibition of plant pathogenic micro-organisms by sequestering iron or producing metabolites with antibiotic properties. Formation of a siderophore complex by the plant may also be involved (Defago and Hass, 1990). Siderophore-mediated competition for iron was indicated as the mechanism of suppression of Fusarium wilt of carnation by *P. putida* (WCS358r) (Duijff *et al.*, 1994) and suppression of phytopathogens to winter wheat (Freitas *et al.*, 1991).

Al-Achi *et al.* (1991) found that, when introduced as pairs into irradiated, sterile soils, a *P. fluorescens* strain prevented optimum colonisation by a *P. putida* strain. The addition of *P. putida* to sterile soil already populated by *P. fluorescens* impeded growth of *P. putida* in that soil. However, adding *P. fluorescens* to soil populated by *P. putida* did not prevent growth of *P. fluorescens* and caused a decrease in *P. putida*. These results suggested competition for similar niches in soils, and that *P. fluorescens* was the more competitive species studied.

#### F. *P. syringae*

Some strains of *P. syringae* have the ability to cause ice nuclei to form at temperatures just below 0°C, thus inducing freezing injury to susceptible plants and allowing disease development to occur (Lindow, 1983). Nutritional starvation for nitrogen, phosphorous, sulphur or iron at 32°C, followed by a shift to 14–18°C, led to the rapid induction (from non-detectable to 100% in 2 to 3 h) of type I ice nuclei (Nemecek-Marshall *et al.*, 1993).

Replacement series experiments on bean leaves between *P. syringae* and epiphytic *P. fluorescens*, *Pantoea agglomerans* (*Erwinia herbicola*), *Stenotrophomonas maltophilia* (*Xanthomonas maltophilia*) and *Methylobacterium organophilum* have demonstrated that the epiphytes were all capable of higher levels of coexistence with *P. syringae* than was observed with another *P. syringae* strain. The level of coexistence with the epiphytes was inversely correlated with the ecological similarity of the strains and with a differential preference for amino acids, organic acids and carbohydrates (Wilson and Lindow, 1994).

The invasion and exclusion abilities of 29 strains of *P. syringae* were studied on leaves in 107 pairwise combinations in which each strain was inoculated on day 0, and the second (challenge) was

inoculated on the same leaf on day 3 (Kinkel and Lindow, 1993). The presence of an established population often significantly reduced the growth of the second strain when quantified on day 6; successful invaders (challenge) were significantly less likely to exclude challenge populations than were non-successful invaders. Hirano and Upper (1993) determined that an introduced antibiotic-resistant strain of *P. syringae* spread but did not persist when applied to bean plants grown in the field; it was concluded that the introduced strain was less fit than the pool of indigenous species. Competition between indigenous soil bacteria and single cells of *P. syringae* pv. *syringae* engineered with bioluminescence genes from *Vibrio harveyi* can be monitored using charge-coupled enhanced microscopy (Silcock *et al.*, 1992).

Defreitas *et al.* (1993) determined that *P. syringae* R25 inoculated on field peas (*Pisum sativum*) did not affect plant growth in plastic growth pouches but, in soil, did inhibit nitrogenase activity of nodules formed by indigenous rhizobia; *P. syringae* R25 inhibited the growth of field beans (*Phaseolus vulgaris*) in both plastic growth pouches and in soil. When peas were inoculated with both *P. syringae* R25 and *Rhizobium leguminosarum*, there was an increase in plant biomass in growth pouches but no effect was observed in soil; when beans were inoculated with both *P. syringae* R25 and *Rhizobium phaseoli*, there were severe deleterious effects on seedling emergence, plant biomass and nodulation in both growth pouches and soil.

Table 4.10 Phytopathogenic strains of *P. syringae* containing plasmids

Pathovar	Reference
<i>P. syringae</i> pv. <i>angulata</i>	Piowarski and Shaw, 1982
<i>P. syringae</i> pv. <i>atrupurea</i>	Sato <i>et al.</i> , 1983
<i>P. syringae</i> pv. <i>coronafaciens</i>	Piowarski and Shaw, 1982
<i>P. syringae</i> pv. <i>glycinea</i>	Curiale and Mills, 1983
<i>P. syringae</i> pv. <i>lachrymans</i>	Coplin, 1989
<i>P. syringae</i> pv. <i>papulans</i>	Burr <i>et al.</i> , 1988
<i>P. syringae</i> pv. <i>phaseolicola</i>	Quant and Mills, 1984
<i>P. syringae</i> pv. <i>savastanoi</i>	Comai <i>et al.</i> , 1982
<i>P. syringae</i> pv. <i>striafaciens</i>	Beck-Von Bodmann and Shaw, 1987
<i>P. syringae</i> pv. <i>syringae</i>	Gonzales <i>et al.</i> , 1984
<i>P. syringae</i> pv. <i>tabaci</i>	Obukowicz and Shaw, 1983; 1985
<i>P. syringae</i> pv. <i>tomato</i>	Denny, 1988; Bender and Cooksey, 1986

### G. *P. tolaasii*

*P. tolaasii* produces a haemolytic lipopeptide toxin, tolaasin, which is a potent biosurfactant (Hutchison and Johnstone, 1993) and the primary disease determinant of its pathogenicity to the cultivated mushroom, *Agaricus bisporus* (Rainey *et al.*, 1993). Tolaasin is also active against a range of basidiomycetes and gram-positive bacteria (Rainey *et al.*, 1991).

The nematode *Caenorhabditis elegans* is reported to decrease the spread of *P. tolaasii* in mushroom growth chambers (Grewal, 1991). *P. fluorescens* biovar *reactans* was frequently isolated from the gut of *C. elegans* along with mushroom sporophores. All the isolates of *P. fluorescens* biovar *reactans* isolated from nematodes were antagonistic to *P. tolaasii*. It was suggested that, as *C. elegans* selects *P. fluorescens* biovar *reactans* rather than *P. tolaasii* as a food, it probably spreads the antagonist in the mushroom crop and may contribute to the control of mushroom blotch (Grewal, 1991). *P. fluorescens* has also been described by other researchers as antagonistic to *P. tolaasii* (Khanna and Olivier, 1989; Munjal *et al.*, 1989; Nair and Fahy, 1972). Nair and Fahy (1972) reported *Enterobacter aerogenes* to be antagonistic to *P. tolaasii*.

Thorn and Tsuneda (1992) report that 23 species of wood-decay basidiomycetes attacked or lysed *P. tolaasii* when tested. Attack took the form of increased hyphal branching within the bacterial colonies, often preceded by directional growth toward them.

## 5. Ability to form Survival Structures (e.g. Spores, Sclerotia)

Pseudomonads are asporogenous, that is, they do not form spores or other survival structures. Pseudomonads are, however, pleomorphic and represent a tremendously diverse group of strains able to tolerate extreme environmental conditions, including the extremes of temperature.

Bacteria that do not form survival structures like spores and cysts are suspected to have other survival strategies. A number of researchers have reported the existence of dwarf or ultramicrobacteria in nutrient-stressed environments (Rosak and Colwell, 1987). These cells have been described from seawater (Amy and Morita, 1983) and soil (Casida, 1977). Cells are able to develop to their full size, once exposed to an abundant supply of nutrients.

## 6. Routes of Dissemination, Physical or Biological

### A. Physical

Pseudomonads may be disseminated by air or water currents. For example, Trevors *et al.* (1990) used soil-core microcosms to study the movement of a *P. fluorescens* isolate through soil planted with wheat and unplanted. In the absence of ground water flow, limited movement was detectable along the soil column planted with wheat, while no movement was detected in the unplanted soil. In contrast, movement of the strain through the column was dependent on the flow rate of the water and the number of times the columns were flushed through. Water flow also affected the distribution of the inoculant along the wheat roots. Bacterial cell size has been related to the movement of cells through a soil column, with smaller bacterial cells (< 1.0 µm) moving fastest through the column (Gannon *et al.*, 1991).

Rain may also be an important source of inoculum and means of dispersal for pseudomonads. Rain splash has been attributed to move pseudomonads colonising leaf surfaces down the plant canopy and into the soil (Hirano and Upper, 1992; Butterworth and McCartney, 1992; McCartney and Butterworth, 1992). Large drops of artificial rain were more effective in dispersing bacteria than smaller drops (Butterworth and McCartney, 1992). Humidity correlated positively with the consequent survival of pseudomonads dispersed by rain splash (McCartney and Butterworth, 1992). However, dispersal is short range (one or a few metres) (Constantidou *et al.*, 1990). Pseudomonads (including *P. syringae* Ice<sup>+</sup> strains) have been found to leave plant surfaces in an aerosol-stable state and enter the troposphere during dry, warm weather (Lindemann *et al.*, 1982; Lindemann and Upper, 1985). They are then transported and washed downwards during rainfall (Constantidou *et al.*, 1990).

### B. Biological

Pseudomonads are motile bacteria characterised by the presence of at least one flagellum. While there is no convincing evidence that the bacteria are flagellated in soil (Stotzky *et al.*, 1991), flagella appear to confer increased epiphytic fitness on *P. syringae* strains in association with moisture on leaf surfaces (Haeefe and Lindow, 1987). The potential for certain fluorescent pseudomonads to colonise plant surfaces has been attributed to the presence of pili (Vesper, 1987; de Groot *et al.*, 1994), surface charge properties (James *et al.*, 1985), the production of agglutinin, a glycoprotein complex, released from root surfaces (Anderson, 1983), and the ability of certain saprophytic pseudomonads to adhere to the agglutinin of specific plant species (Glandorf *et al.*, 1993; 1994).

Earthworms moving through soil have been implicated in the dissemination of bacteria over short distances. As well, Johnson *et al.* (1993) have demonstrated the ability of honey bees to disseminate a biological control strain of *P. fluorescens* used against the fireblight pathogen, *Erwinia amylovora*, in apple and pear blossoms. Honey bees carrying approximately  $10^4$  to  $10^6$  cfu per bee effectively inoculated fruit tree blossoms with bacteria.

## 7. Containment and Decontamination

Containment plans have been proposed for microbial releases, although few of them have been used, and their efficacy is yet to be demonstrated. It is likely to be difficult to eliminate all the bacteria from a site of introduction. Many of the proposed chemical treatments have gross rather than localised effects; hence their application may have considerable impact on the natural flora, fauna and microflora at the site. Pseudomonads will colonise many laboratory and hospital disinfectants, and may exhibit broad spectrum resistance to a number of widely used antibiotics. Disinfectants based on quaternary ammonium compounds and chlorhexidine solutions have been found to be contaminated with pseudomonads. Disinfectant contaminants include *P. aeruginosa*, *P. fluorescens*, and *P. cepacia* (Bergen, 1981).

*P. putida* strains that degrade alkylbenzoates have been modified to carry a fusion of the P (lac) promoter to the *gef* gene, which encoded a killing protein (Molin *et al.*, 1993; Ramos *et al.*, 1994). Expression from P (lac) was controlled through a regulatory cascade, so that P (lac) was switched on or off by the absence or presence of alkylbenzoates respectively. Similar uncontained strains were also constructed and tested as a control. Contained and uncontained strains were genetically stable, and their survival and functionality in soil microcosms were as expected. Both contained and uncontained strains survived well in soils supplemented with alkylaromatics, whereas survival of the contained strain in soil microcosms without methylbenzoates was markedly reduced in contrast to the control strain, which survived in these soils in the absence of alkylbenzoates (Jensen *et al.*, 1993; Ronchel *et al.*, 1995).

## 8. Description of Detection and Monitoring Techniques, Including Specificity, Sensitivity and Reliability

### A. Techniques employed in the laboratory and/ or environment for detecting the presence of, and for monitoring, numbers of the organism

Information on detection and monitoring techniques is provided in this information element as well as in information element 2 and Table 4.11. Each of the well-described detection methods has limitations as well as advantages for enumeration and/or detection (Drahos, 1992). For example, under certain conditions an approach which provides reasonable sensitivity by culturing a micro-organism (*e.g.* the viable plate count) may give reliable data for culturable populations. Furthermore, many approaches are complementary; methods utilising nutritional, antibiotic and enzymatic markers rely on the ability of the target organism to express the marker genes during the selection or reculturing process. However, expression of these traits may not always be optimal, for instance under conditions of severe environmental stress. In these situations, a direct method of detection could be used.

#### *Selective plating*

Selective plating has been used widely in combination with selectable phenotypes based on antibiotic resistances (often spontaneous mutations) (Compeau *et al.*, 1988; Fredrickson *et al.*, 1989; Thompson *et al.*, 1990) or introduced genes such as *xylE* (Winstanley *et al.*, 1989; Morgan *et al.*, 1989) *lacZY* (Cook *et al.*, 1991; Drahos *et al.*, 1988), *lux* (Shaw and Kado, 1986) and *mer* gene (Iwasaki *et al.*, 1993; 1994). A number of these genes have been used for marking and tracking pseudomonads. It is important to ensure

that the marker is not found in the indigenous microflora of the environment to which the micro-organism will be introduced.

*Pseudomonads* appear to be highly culturable on laboratory media and may be isolated from environmental samples using viable plating (Drahos, 1992). Generally, 1 g of environmental sample is homogenised or shaken in 9 ml of an appropriate diluent such as ¼ strength Ringer's solution or physiological saline. The homogenate is serially diluted 1 in 10, 100 µl aliquots spread onto selective agar, and the plates incubated at 28°C. A number of selective media are available commercially, such as *Pseudomonas* selective agar (Oxoid) and *Pseudomonas* agar F (Difco). Both media have a low iron content, promoting the production of the iron-chelating, fluorescent siderophores. Selective agars are supplemented with antibiotics. A commercially prepared cocktail of cephaloridine, fucidin acid and cetrimide (Oxoid) is available which may be supplemented with ampicillin and the antifungal agent, cyclohexamide. Micro-organisms may be detected at or above a detection limit of  $10^2$  (*i.e.* one cell may be detected when a minimum of 100 are present per g of sample) (Trevors and van Elsas, 1989). Sensitivity may be increased by plating larger volumes or by using smaller dilutions, *i.e.* 1 in 2 instead of 1 in 10.

#### ***Most probable number***

Most probable number (MPN) methods (Alexander, 1982) have been used to attain greater sensitivity. A serial dilution of the sample is made in an appropriate diluent to an extinction point (Atlas, 1982). Three to ten replicates of each dilution are made and the pattern of positive and negative scores recorded (*i.e.* growth or no growth). Statistical tables are used to determine the MPN of micro-organisms present in the sample. MPNs like the viable plate count require growth and reproduction of the strains, and may be less accurate since an MPN is established with confidence limits (Jain *et al.*, 1988).

**Table 4.11 Examples of identification and detection techniques**

Method	Reference	Sensitivity/reliability
DNA extraction followed by Polymerase Chain Reaction (PCR)	Stefan and Atlas, 1988	100 <i>P. cepacia</i> cells 100g <sup>-1</sup> sediment, against a background of 10 non-target organisms
	Pillai <i>et al.</i> , 1991	1 to 10 <i>E. coli</i> (with <i>Tn5</i> insert) colony forming unit (cfu)g <sup>-1</sup> soil
	Tsai and Olson, 1992	3 cells <i>E. coli</i> g <sup>-1</sup> soil; primers directed at 16S rRNA
	Tushima <i>et al.</i> , 1995	10 cells g <sup>-1</sup> water
hybridisation using radio-labelled probes	Holben <i>et al.</i> , 1988; Stefan and Atlas, 1988	10 <sup>3</sup> to 10 <sup>4</sup> cells g <sup>-1</sup> soil
	Jain <i>et al.</i> , 1988; Blackburn <i>et al.</i> , 1987	10 <sup>2</sup> cells g <sup>-1</sup> soil (similar to viable plate count)
direct microscopy using immuno-fluorescence	Schmidt, 1974; Bohool and Schmidt, 1980; Ford and Olson, 1988	10 <sup>6</sup> to 10 <sup>7</sup> cells g <sup>-1</sup> soil
enzyme-linked immunosorbent assays (ELISA)	Morgan <i>et al.</i> , 1991; Scholter <i>et al.</i> , 1992	10 <sup>1</sup> cells g <sup>-1</sup> soil; 10 <sup>-10</sup> cells g <sup>-1</sup> soil
selective viable plating	Trevors and van Elsas, 1989; Iwasaki <i>et al.</i> , 1993, 1994	10 <sup>2</sup> cfu g <sup>-1</sup> soil; 1 cfu ml <sup>-1</sup> water; 10 cfu g <sup>-1</sup> soil
most probable number (MPN) viable counts	Alexander, 1982; De Leij <i>et al.</i> , 1993	< 10 <sup>2</sup> cfu g <sup>-1</sup> soil; < 10 <sup>1</sup> cfu g <sup>-1</sup> soil

### Simple chemotaxonomical approach

A simple chemotaxonomical approach which avoids isolation and cultivation of micro-organisms has been used. For example, quinone profiles (Hiraishi *et al.*, 1991) or polyamine patterns (Auling *et al.*, 1991) have been used as biomarkers for a survey of pseudomonads (and acinetobacters) in activated sludge from sewage treatment facilities.

### Immunological methods

SDS-PAGE coupled with immunological probes have been applied to identify fluorescent pseudomonads of environmental origin (Sorenson *et al.*, 1992). Other possibilities for detecting pseudomonads in environmental samples include the application of phylogenetic probes applied in situ hybridisations (DeLong *et al.*, 1989), or strain or species-specific monoclonal antibodies labelled with fluorescent dyes (Bohloul and Schmidt, 1980; Conway de Macario *et al.*, 1982). Blair and McDowell (1995) describe an ELISA method for detecting extracellular proteinase of *P. fragi*.

Microscopic examination and direct enumeration of micro-organisms *in situ* can also be used, although this type of approach is not sensitive. To detect one bacterium at a magnification of 1000, the cell density must be 10<sup>6</sup> to 10<sup>7</sup> per g soil. The approach does, however, provide information about the spatial distribution of a strain colonising an environmental substrate, and can be used to enumerate non-culturable micro-organisms.

Ramos-Gonzalez *et al.* (1992) produced highly specific monoclonal antibodies against surface lipopolysaccharides (LPS) of *P. putida* 2440 and developed a semi-quantitative dot blot immunoassay for bacteria in liquid media. This allowed the authors to detect, in complex samples, as few as 100 cells per spot by using peroxidase-conjugated antibody against the antibody that recognised *P. putida* 2440. An intrinsic limitation of this technique is the turbidity of the samples, which may limit maximum assay volume. This assay is also of limited use for bacteria introduced into soils or sediments because of intrinsic fluorescent backgrounds. *P. putida* 2440 (pWWO) released in lake mesocosms have been successfully tracked with monoclonal antibodies (Brettar *et al.*, 1994; Ramos-Gonzalez *et al.*, 1992).

### ***Nucleic acid probes and primers***

Nucleic acid probes and/or PCR primers may be used for the detection of gene sequences in the environment. A number of sequence hybridisation techniques including Southern, slot-blot, dot-blot, and colony hybridisation have been used for environmental isolates. These approaches would be particularly applicable to strains with traits that are not widely distributed throughout the environment under study, and against which specific probes and primers may be designed. The sensitivity of the hybridisation approach is variable and for the most part strain-specific. Generally, radioactively labelled probes provide for more sensitivity than non-radioactive probes. Sensitivity can be enhanced using PCR. However, the increased efficiency of the amplified signal obtained by the PCR assay is countered by the inefficient extraction of nucleic acids from environmental samples (Bramwell *et al.*, 1994). For example, soils contain positively charged cations which are sandwiched between layers of clay, and which are able to bind negatively charged nucleic acids, making their retrieval difficult. Caution is required in using PCR as a method for the enumeration of bacteria, as the extreme sensitivity of this procedure renders quantification by target dilution difficult (Drahos, 1992). Thiem *et al.* (1994) and Zhou and Tiedje (1995) point out the complexity of using molecular techniques for monitoring pseudomonads used for subsurface bioremediation.

Denaturing gradient gel electrophoresis (DGGE) of DNA is a suitable method for those species which are difficult to culture on growth media. This method has been used by Muyzer *et al.* (1993). Whole DNA is isolated. Using two primers, one with a GC-rich end, a fragment of 16S rDNA is amplified by PCR. This results in a mixture of DNA fragments, equal in size but different in sequence, corresponding to the various organisms in the sample. The mixture is fractionated by DGGE, resulting in one band for each organism type. The bands are sequenced, and based on the sequences, the rRNA-group can be determined.

### ***Polymerase chain reaction (PCR) based sequence amplification***

A technique that is finding increasing application for specific identification of micro-organisms is the technique referred to as REP-PCR (based on PCR amplification between repetitive sequences commonly found in bacteria). This technique relies on development of adequate databases, but is used with increasing frequency (De Bruijn, 1992). Other approaches are to follow the expressed phenotype attributed to the introduction of a marker gene (*e.g.* bioluminescent genes) (Prosser, 1994), and to use competitive PCR based on introduction of an internal standard during the PCR amplification (Leser, 1995).

### ***Arbitrary PCR primers***

Identification can be facilitated based on the analysis of DNA produced from total DNA, using PCR and arbitrary primers (Welsh and McClelland, 1990; Williams *et al.*, 1990).

### ***Specific PCR primers***

*P. aeruginosa* can be identified using PCR amplification of the 16S-23S rDNA internal transcribed spacer region (Tyler *et al.*, 1995).

## B. Specificity, sensitivity, reliability

The specificity of identification/monitoring methodologies will generally require some sort of experimental study to demonstrate that the method distinguishes the introduced inoculant from indigenous relatives. An approximate estimate of sensitivity for a number of methods is given in Table 8. However, sensitivity of detection is a function of the organism and of the habitat.

A problem with applying any method of detection is its dependence on extraction efficiencies. Problems are exemplified in soil. Traditionally, bacteria have been recovered from soils through the mechanical shaking of the soil in an appropriate diluent. The ease of extracting cells or nucleic acids varies between soil types, with extraction efficiencies being higher in sand as opposed to clay-based soils. Strong chemical and physical interactions may occur between micro-organisms and the particulate matter of soil. These associations may be ionic, since bacteria are negatively charged and clay soil minerals contain positively charged cations. Dispersion of soil aggregates has been considered important, as entrapment of micro-organisms in soil aggregates is considered to be one of the most significant means by which micro-organisms are retained in soil (Hopkins *et al.*, 1991). Attempts to disrupt these soil-microbe associations to extract bacteria have utilised homogenisation, chemical dispersants, cation exchange resins, and differential centrifugation (Faegri *et al.*, 1977; Bakken, 1985; MacDonald, 1986; Herron and Wellington, 1990; Hopkins *et al.*, 1991).

Soil is a highly heterogeneous substrate with a non-uniform spatial distribution of bacterial colonies (Wellington *et al.*, 1990). Sampling strategies should consider the variability of the soil matrix under study; errors attributable to the difficulties of sampling heterogeneous substrates may be compensated for by taking composite samples (Atlas and Bartha, 1981).

Micro-organism themselves will also affect the efficiency of extraction of biological molecules such as DNA. For example, bacteria, even those quite closely related, vary in the conditions required for lysis. Hence methodologies aiming to extract the total DNA from soil will selectively recover DNA from isolates that lyse easily, making representative sampling of environmental substrates difficult.

Similar selective pressures apply to viable plating methodologies, since these methods favour the growth of bacteria that readily grow on agar plates under laboratory conditions. Furthermore, all media are selective to some extent, so that certain bacterial species will appear in different proportions, if at all, on different bacteriological agars. Sorheim *et al.* (1989) compared the populations recovered from soil on three different non-selective media. Bacterial populations exhibiting the same level of diversity were isolated on all media. Each of the media appeared to select for a different population of isolates, with 30% of the population appearing common to all three media. 20% of the isolates recovered from two of the media were distinct to that particular media, and 60% of isolates on the third media were unique to it.

The sensitivity of the viable plate count has been estimated to be  $10^2$  cfu/g soil (Trevors and van Elsas, 1989). However, this may be improved by combining methods to extract and concentrate the biomass from environmental material prior to plating. Detection limits as low as 10 streptomycete spores per 100 g sterile soil have been demonstrated (Herron and Wellington, 1990).

Pseudomonads are highly culturable on rich media. Their importance may therefore have been overestimated as a result of over-representation on isolation plates (Miller *et al.*, 1990b; Sorheim *et al.*, 1989). Nutritionally limiting isolation media and lower incubation temperatures with longer incubations may allow a greater diversity of bacterial isolates to be recovered from environmental substrates (Miller *et al.*, 1990b).

Ottawa' 92: *The OECD Workshop on Methods for Monitoring Organisms in the Environment* (OECD, 1994a) includes a review of the monitoring of micro-organisms (including *P. aureofaciens*) in the phyllosphere (Bailey *et al.*, 1994) and a review of the different methods available. A companion document, *Compendium of Methods for Monitoring Organisms in the Environment* (OECD, 1994b), contains 39 methods for detecting or monitoring micro-organisms, including the following species of *Pseudomonas*: *P. aureofaciens*, *P. fluorescens*, *P. putida*, *P. aeruginosa* and *P. syringae*.

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## **SECTION 3**

### **ACIDITHIOBACILLUS**

#### **I. General Introduction**

This document presents information that is accepted in the literature about the known characteristics of bacteria in the genus *Acidithiobacillus*. Regulatory officials may find the technical information useful in evaluating properties of micro-organisms that have been derived for various environmental applications. Consequently, this document provides a wide range of information without prescribing when the information would or would not be relevant to a specific risk assessment. The document represents a snapshot of current information (end-2002) that may be potentially relevant to such assessments.

In considering information that should be presented on this taxonomic grouping, the Task Group on Micro-organisms has discussed the list of topics presented in the “Blue Book” (*i.e.* Recombinant DNA Safety Considerations (OECD, 1986)) and attempted to pare down that list to eliminate duplications as well as those topics whose meaning is unclear, and to rearrange the presentation of the topics covered to be more easily understood (the Task Group met in Vienna, 15-16 June, 2000). This document is a first draft of a proposed Consensus Document for environmental applications involving organisms from the genus *Acidithiobacillus*.

#### **II. General Considerations**

##### **1. Subject of Document: Species Included and Taxonomic Considerations**

The four species of *Acidithiobacillus* covered in this document were formerly placed in the genus *Thiobacillus* Beijerinck. In recent years several members of *Thiobacillus* were transferred to other genera while the remainder became part of three newly created genera, *Acidithiobacillus*, *Halothiobacillus*, *Thermithiobacillus*, and to the revised genus *Thiobacillus sensu stricto* (Kelly and Harrison, 1989; Kelly and Wood, 2000). The reassignment to these three newly designated genera was based on physiological characteristics and 16S rRNA gene sequence comparisons (Kelly and Wood, 2000). *Acidithiobacillus* contains two species (*A. ferrooxidans* and *A. thiooxidans*) of the original genus *Thiobacillus* that have the potential to cause significant ecological damage. Two other species have been reassigned to this new genus, *A. caldus* and *A. albertensis*. All these species have been, or are likely to be, employed in various biotechnological applications in the environment.

##### **2. Characteristics of the Organism: Identification and the Methods Used to Identify the Organism**

###### **A. Characterisation of the genus *Acidithiobacillus***

The genus was established by Kelly and Wood (2000), with *A. thiooxidans* (formerly *Thiobacillus thiooxidans*) as the type species. The four species included in the genus are Gram-negative, rod-shaped ( $0.4 \times 2.0 \mu\text{m}$ ), motile with one or two flagella, and possess the ability to use reduced sulphur compounds as electron donor for autotrophic growth, in common with various other unrelated “sulphur bacteria” (Kelly and Harrison, 1989; Kuenen *et al.*, 1992). As with other *Thiobacillus* species now redistributed, members of this genus are distinguished morphologically from other colourless sulphur bacteria by

forming external rather than internal sulphur particles (Kuenen, 1989). They are strictly aerobic and obligately acidophilic (optimum pH < 4.0). Some species oxidise ferrous iron and hydrogen (Table 4.12) or use natural and synthetic metal sulphides to generate energy, while one species (*A. ferrooxidans*) can oxidise iron. The optimum temperature ranges from 30–35 °C for mesophilic species to 45 °C for moderately thermophilic species. All of the species contain ubiquinone Q-8, and the G+C content of the DNA is 52–64 mol %. *Thiobacillus sensu stricto* now contains only species belonging to the  $\beta$ -subclass of the Proteobacteria, but *Acidithiobacillus*, together with *Halothiobacillus* and *Thermithiobacillus*, have been assigned to the  $\gamma$ -subclass (Kelly and Wood, 2000). A full account of the genus is given in the section contributed by Kelly and Wood (2005) in the 2<sup>nd</sup> edition of Bergey's Manual of Systematic Bacteriology.

### B. Differentiation of *Acidithiobacillus* from related taxa

Members of the genus are distinguished by their obligate acidophilic nature (pH < 4.0) and possession of ubiquinone Q-8.

### C. Characters used in classification

#### *Phenotypic characters*

Many of the phenotypic characters of *Acidithiobacillus* such as the rod-like shape, motility, Gram-negative reaction and utilisation of sulphur compounds are shared in common with species formerly placed in *Thiobacillus*. These characters are useful for broad recognition but no longer for critical identification.

#### *Mol% G+C content*

The determination of the mol % G+C content of the DNA of bacterial isolates has been used for a long time to determine whether strains could be related to each other. It is to some extent, a negative test. While widely differing G+C values can suggest that two isolates are not related, matching G+C values do not guarantee that they are the same. G+C values for the four species of *Acidithiobacillus* are, however, often sufficiently far apart to serve as useful species characteristics.

#### *Ubiquinones and cellular fatty acid analysis*

Lane *et al.* (1985) determined that there was a correlation between ubiquinone type and physiological behaviour. Katayama-Fujimura *et al.* (1982) used types of ubiquinones and the DNA base composition to differentiate 11 species of the former genus *Thiobacillus*. The association between species and ubiquinone type was constant except for *T. perometabolis*, one strain having 8 and the other 10-isoprene units. Species presently assigned to *Acidithiobacillus* all possessed eight units. The strain of *A. ferrooxidans* examined by the later authors was unique in that it had ubiquinones with 9 as well as 8 isoprene units.

#### *Nucleotide structure*

5S rRNA sequences were obtained for thirteen species of the original genus *Thiobacillus* (Lane *et al.*, 1985, 1992) and these sequences were shown to be distinct for each species. Similarities within the sequences also enabled the species also to be assigned to the  $\alpha$ ,  $\beta$  or  $\gamma$  groups of the four groups of Proteobacteria, the last group including species of *Acidithiobacillus*.

#### *DNA homologies*

DNA hybridisation studies on *A. ferrooxidans* and *A. thiooxidans*, together with *Thiobacillus thioparus* and five bacteria formerly placed in *Thiobacillus* (Huber and Stetter, 1989, 1990) established the value of these tests because they showed that there was usually a high degree of homology (>70%) between strains of the same species.

Table 4.12 *Acidithiobacillus* : Characters used in classification

Species	Optimum pH <sup>a</sup>	pH range	Optimum temperature	Temperature range	Ubiquinone	Mol % G+C	Subclass of Proteobacteria	References
Strictly chemolithotrophic and autotrophic								
<i>Acidithiobacillus albertensis</i>	3.5-4.0	2.0-4.5	28-30	ND	Q-8	61-62	ND	b, c
<i>Acidithiobacillus ferrooxidans</i>	2.0-2.5	1.3-4.5	30-35	2-37	Q-8	58-59	$\gamma$	b, d, e
<i>Acidithiobacillus thiooxidans</i>	2.0-3.0	0.5-5.5	28-30	10-37	Q-8	52	$\gamma$	b, f
Facultatively chemolithotrophic or mixotrophic with tetrathionate								
<i>Acidithiobacillus caldus</i> <sup>1</sup>	2-2.5	1-3.5	45	32-52	Q-8	63-64	$\gamma$	g

ND: not determined

<sup>1</sup>Moderately thermophilic<sup>a</sup>Katayama-Fujimura *et al.* (1982); <sup>b</sup>Kelly and Harrison (1989); <sup>c</sup>Bryant *et al.* (1983); <sup>d</sup>Leduc and Ferroni (1994); <sup>e</sup>McCready (1988); <sup>f</sup>Fliermans and Brock (1972); <sup>g</sup>Hallberg and Lindström (1994).

#### D. Comments on the species

##### *Acidithiobacillus albertensis*

Syn. *Thiobacillus albertensis* (Bryant *et al.*, 1983; Kelly and Harrison, 1989; Kelly and Wood, 2000). This species is distinguished morphologically by a tuft of polar flagella and a glycocalyx extending outwards from the outer membrane of the bacterial cell envelope and which is used to attach itself to elemental sulphur (Bryant *et al.*, 1983). These features together with the relatively high G+C content of the DNA, differentiate this species from the other three (Kelly and Wood, 2000). It has been tentatively assigned to *Acidithiobacillus* because a 16S rRNA sequence for the species is not yet available (Kelly and Wood, 2005).

##### *Acidithiobacillus caldus*

Syn. *Thiobacillus caldus* (Hallberg and Lindstrom, 1994; Kelly and Harrison, 1989; Kelly and Wood, 2000). This species is distinguished by extremely short rod-shaped cells, each with a single polar flagellum (Hallberg and Lindstrom, 1994), and by its moderately thermophilic nature. It cannot oxidise sulphidic ores, but it may be found associated with others involved in leaching. It is facultatively rather than obligately chemolithotrophic. A specific, fast and sensitive non-radioactive immuno-binding assay had been used for the detection and enumeration of this species (Amaro *et al.*, 1994). Chemiluminescence or peroxidase-conjugated immunoglobulins are employed in a dot or slot blotting system. This method is very convenient for monitoring bioleaching micro-organisms in effluents from industrial bioleaching processes.

##### *Acidithiobacillus ferrooxidans*

Syn. *Thiobacillus ferrooxidans* (Temple and Colmer, 1951; Kelly and Harrison, 1989; Kelly and Wood, 2000). Morphologically this species appears to be distinguished by a single coiled flagellum in mature cells (Gonzalez and Cotoras, 1987). Also, this is the only species in the genus so far to be able to utilise iron as well as sulphur.

Serological and electrophoretic methods have been employed for the rapid detection of isolates of *A. ferrooxidans* and the differentiation of strains (Jerez *et al.*, 1986). Different serotypes, characterised by specific lipopolysaccharide banding patterns in polyacrylamide gels, have been described (Koppe and Harms, 1994). A specific and very sensitive dot-immuno-binding assay for the detection and enumeration of *A. ferrooxidans* has been developed by Arredondo and Jerez (1989). Samples were spotted onto nitrocellulose membranes and first incubated with polyclonal antisera, derived from a rabbit inoculated with whole cells of *A. ferrooxidans*, and in <sup>125</sup>I-labeled protein A or <sup>125</sup>I-labelled goat anti-rabbit immunoglobulin G. The membranes were then dried, autoradiographed on Fuji Rx X-ray film and scanned at 550 nm. The antisera reacted with every strain of *A. ferrooxidans* tested but not with *A. thiooxidans* and *H. neapolitanus* and three species formerly placed in *Thiobacillus*.

A specific, fast and very sensitive immuno-electron microscopy method was also developed to identify *A. ferrooxidans* present with other iron oxidising bacteria in acidic mine waters (Coto *et al.*, 1992). Polyclonal antisera, produced against whole cells of *A. ferrooxidans*, *A. thiooxidans* and *Leptospirillum ferrooxidans* gave highly specific reactions when cross-reacted with 23 strains of acidophilic bacteria using an immuno-fluorescence staining technique (Koppe and Harms, 1994). These methods have been criticised, however, because of the inability of the antisera to distinguish between dead and living cells (Khalid *et al.*, 1993).

A systematic study of a large collection of strains ascribed to *A. ferrooxidans* revealed considerable diversity among them (Harrison, 1982). The members of seven DNA homology groups recognised by

Harrison (1982) were largely homologous with strains inside each group but to a lesser degree with strains in other groups. Although all strains grew between 25 and 30 °C, some were able to grow at 5 °C and others at 40 °C, and two genomic groups could be distinguished by different temperature optima. The strains in two other genomic groups (1 and 7) were apparently unable to use elemental sulphur, and their high mol% G+C values (53 and 65 respectively) were well outside the range normally accepted (see Table 4.12), giving rise to the suspicion that they might represent different species. Although strain m-1, comprising the seventh genomic group, was later shown to be able to oxidise elemental sulphur on prolonged incubation (Johnson, 1995b), it has been further distinguished from a more typical strain of *A. ferrooxidans* by its classification in the 5S rRNA sequence group III rather than in group II (Lane *et al.*, 1985).

### *Acidithiobacillus thiooxidans*

Syn. *Thiobacillus concretivorus* Parker *Thiobacillus thiooxidans* (Waksman and Joffe, 1922; Kelly and Harrison, 1989; Kelly and Wood, 2000). This species is motile by means of a single polar flagellum, and as with *A. caldus*, it cannot oxidise iron or pyrite, although it can grow on sulphur from pyrite in conjunction with *Leptospirillum ferrooxidans* (Kelly and Wood, 2000). In a study by Harrison (1982), four strains of *A. thiooxidans* were found to be largely homologous with a fifth strain but not with representatives of the seven homology groups of *A. ferrooxidans*. However, a sixth strain ascribed to *A. thiooxidans* showed no similarity to any of them. This indicates that there may be atypical representatives of *A. thiooxidans* as well as *A. ferrooxidans*.

### 3. Information on the Organisms' Reproductive Cycle (Sexual/ Asexual)

*Thiobacillus sensu lato*, in which *Acidithiobacillus* was included, was found to reproduce by binary cell division (Sokolova and Karavaiko, 1968). No spores were observed.

### 4. Biological Features and Environmental Conditions Which Affect Survival, Reproduction, Growth, Multiplication or Dissemination

#### A. Growth requirements

Most strains are able to produce colonies on appropriate media solidified with agar or agarose. The use of elemental sulphur is avoided because of its insolubility. Formation of hydrogen sulphide is potentially toxic in moderate concentrations, and the most widely used sulphur compound is thiosulphate (Smith and Strohl, 1991).

Some strains, especially those of *A. thiooxidans*, grow poorly on agar media, possibly due to the toxicity of agar hydrolysis products (Kelly and Harrison, 1989). The problem is generally solved by the use of a minimal concentration of agar, screening for suitable brands of purified agars, use of agarose, and, in the case of *A. thiooxidans*, a combination of low agar concentration with pH 2.2-2.5 and ferrous sulfate at only about 20 mM (Johnson, 1995a). The growth of strains of *A. ferrooxidans* on solid media is also difficult. However, the double-layered plates such as FeTSBo and described by Johnson (1995a) allow also the growth of most strains.

Species of *Acidithiobacillus*, in common with other former species of *Thiobacillus*, are able to use carbon dioxide as the sole source of carbon for synthesis of cell material (Kuenen, 1975). Ribulose diphosphate carboxylase, the enzyme responsible for the fixation of carbon dioxide, appears to be located in polyhedral inclusions in the cell.

In contrast to species of *Halothiobacillus* which have been recorded as either halotolerant or having a strict NaCl requirement (Sievert *et al.*, 2000), *A. ferrooxidans* has been recorded as unable to grow at salt

concentrations above 1% (Lazaroff, 1963; McCready, 1987; Razzell and Trussell, 1963) whereas specific information for other species of *Acidithiobacillus* for their tolerance to salt is not available.

*Acidithiobacillus ferrooxidans* has a remarkable physiology that allows it to thrive in an inorganic mining environment. Its minimum growth requirements can be satisfied by water, air, an oxidisable iron or sulphur source and trace minerals. The trace elements required are usually present as impurities in the water or ore (Rawlings and Woods, 1995). This statement is also true for *A. thiooxidans*, however elucidation of these abilities for other members of *Acidithiobacillus* is still not available. *A. caldus* was found to be relatively insensitive to a number of xanthate and dithiocarbamate-based flotation reagents but sensitive to a number of mercapto-benzthiazole-based reagents (Okibe and Johnson, 2002).

## B. Oxidation of hydrogen

In contrast to strains of *A. thiooxidans*, *H. neapolitanus*, *T. prosperus*, *Leptospirillum ferrooxidans* and four species formerly placed in *Thiobacillus*, three strains of *A. ferrooxidans* were found to be facultative hydrogen oxidisers, being able to use molecular hydrogen as a sole source of energy (Drobner *et al.*, 1990). The ability to oxidise hydrogen was repressed by ferrous iron or sulphur and occurred only in the presence of oxygen.

## C. Nitrogen

In addition to their ability to fix carbon dioxide all strains of *A. ferrooxidans* examined so far are also able to fix atmospheric nitrogen (*i.e.* they are diazotrophic)(Rawlings and Kusano, 1994).

## D. Aerobic/Anaerobic growth

*Acidithiobacillus* species are strict aerobes with the exception of *A. ferrooxidans*, which is a facultative aerobe. In the absence of oxygen, *A. ferrooxidans* is able to grow on reduced inorganic sulphur compounds using ferric iron as an alternative electron acceptor (Pronk *et al.*, 1992; Sugio *et al.*, 1985).

## E. Resistance to metals

Resistance to metal ions is a function of those thiobacilli tested to date. *Acidithiobacillus ferrooxidans* is resistant to a variety of metal ions such as chromium (Baillet *et al.*, 1998), copper, zinc, nickel, thorium and uranium (Leduc *et al.*, 1997; Tuovinen *et al.*, 1971) and mercury (Takeuchi *et al.*, 1999, 2001; Sugio *et al.*, 2001). According to Iwahori *et al.* (2000), the resistance of *A. ferrooxidans* to mercury is ferrous iron dependent.

## F. Role of Rusticyanin

Rusticyanin is a blue copper protein present in the periplasmic space of *A. ferrooxidans*. Consisting of a single polypeptide chain with one copper atom as a cofactor (Hazra *et al.*, 1992), rusticyanin reportedly serves as the initial electron acceptor upon oxidation of ferrous iron (Hazra *et al.*, 1992; Hutchins *et al.*, 1986). An acid-stable cytochrome *c* was found to catalyse the reduction of rusticyanin (Blake *et al.*, 1988).

## G. Survival

No information on factors influencing survival of *Acidithiobacillus* in the natural environment appears to be available. According to Kelly and Harrison (1989), *A. ferrooxidans* survives in culture on pyrite (FeS<sub>2</sub>) for very long periods when stored at 5-15 °C. Many strains have been successfully freeze-dried or have survived storage in liquid nitrogen or in glycerol suspension at -20 °C (Kelly and Harrison, 1989).

Hubert *et al.* (1994) have shown that survival rates of *A. ferrooxidans* decreased rapidly under laboratory conditions above and below the individual temperature ranges of psychrotrophic and mesophilic strains.

## H. Adhesion

The ability to adhere to surfaces seems to be a peculiar feature of *Acidithiobacillus*. Myerson and Kline (1983) observed the physical adsorption of cells of *A. ferrooxidans* to the surface of different non-porous solid particles (glass, pyrite, sulphur). Selective adherence to iron containing minerals appears to occur naturally (Ohmura *et al.*, 1993), and the ferrous ion, but not the ferric ion, inhibited such selective adhesion. A model of the biofilm structure has been proposed by Karamanev (1991). Intimate contact and adhesion are required for enzymatic attack by *A. ferrooxidans* on insoluble substrates such as sulphur, pyrite ( $\text{FeS}_2$ ), and chalcocopyrite ( $\text{CuFeS}_2$ ), and this is brought about by either a proteinaceous surface appendage (Devasia *et al.*, 1993) or by extra-cellular polymeric substances (specifically lipopolysaccharides) combined with iron (III) (Gehrke *et al.* 1998, 2001; Blais *et al.*, 1994). *Acidithiobacillus ferrooxidans* does not randomly adsorb onto pyrite or other surfaces, but congregates selectively at sites where dislocations, grain boundaries and other non-uniformities in the crystal structure emerge to the surface (Andrews, 1988; Bagdigian and Myerson, 1986; Gehrke *et al.*, 1998). It is possible that diffusion of sulphur atoms along dislocations in the substrate is an important part of the mechanism of microbial decomposition. This pattern of diffusion provides a great advantage to bacteria because sulphur oxidation has a much higher yield of free energy than iron oxidation. No corresponding advantage would be gained by adsorption onto pure pyrite sites because the diffusion through pyrite crystals is several orders of magnitude lower.

Growth of bacteria adhering to the mineral surface initiates the oxidation process in arsenopyrite bioleaching (Fernandez *et al.*, 1995). Corrosion patterns appear, with the liberation of ferrous ions and formation of elemental sulphur. With the increase in number of the bacteria, the ferrous ions are oxidised to ferric ions with the ultimate production of ferric arsenate (Fernandez *et al.*, 1995).

Adhesion structures, consisting of a filamentous matrix, have been observed in *A. thiooxidans* (Blais *et al.*, 1994), linking the cells to the surface of sulphur particles. They were not observed however in *T. thioparus*, where the cells fixed directly onto the sulphur (Sokolova and Karavaiko, 1968). The latter process is the usual type of fixation observed in the species placed formerly in *Thiobacillus*.

Adhesion can be estimated using the technique of Dziurla *et al.* (1998), who developed an immunofiltration assay (ELIFA) for this purpose. ELIFA is a modified ELISA using micro-titer plates with 0.2- $\mu\text{m}$  pore-size filters in place at the bottom. Particles, either previously inoculated with bacteria or to be reacted with added bacteria are incubated in the wells and then successively filtered and washed by applying a vacuum to the bottom of the plate. The inoculated particles are retained by the filter. Polyclonal antiserum raised against a strain of *A. ferrooxidans* is added and the plates incubated at 35 °C for one hour. The polyclonal rabbit antibody used was shown to react with different *A. ferrooxidans* and *A. thiooxidans* but not with other bacterial genera. Following washing the bound antibody is detected with goat-anti-rabbit globulin conjugated to alkaline phosphatase and p-nitrophenylphosphate was used as the detection substrate.

## I. pH and nutrition preference

Species of *Acidithiobacillus* are acidophilic as well as obligate or facultative chemolithotrophs according to the nutritional table presented by Kuenen *et al.* (1992). Species of *Acidithiobacillus* are abundant in acid mine drainage water where they oxidise and gain energy from the oxidation of metals such as iron. The optimum pH for *A. ferrooxidans* is between 2-3, but when the substrate is in large part pyritic, the pH can reach extremely low values, (less than 1). This is due to the availability of abundant

sulphur and the precipitation of ferric hydroxide when the solution reaches saturation (Morin, 1995). *Acidithiobacillus caldus* is the single mixotrophic species which can utilise sulphur or tetrathionate and yeast extract or glucose (Hallberg and Lindstrom, 1994).

Blais *et al.* (1993) have demonstrated that less acidophilic bacteria in sludge such as *Thermithiobacillus tepidarius*, *T. aquaesulis*, *T. denitrificans*, *T. thioparus* and other species formerly placed in *Thiobacillus*, may initiate the acidification to the point where the acidophilic species can take over. Acidophilic bacteria decreased the pH of a sulphur-containing synthetic salts medium to the level of 1.4-1.6 in 10 days. Evangelou (1995) mentioned pH 3.5 as the upper limit below which  $\text{Fe}^{2+}$  is oxidised by *A. ferrooxidans*. For the less acidophilic bacterial species, the limit of acidification was more variable, between pH 2.2 and 6.9. This has important implications for the removal of heavy metals from sludge (presented later in this document).

#### J. Temperature relations

*Acidithiobacillus* contains one thermophilic species, *A. caldus*, but little is known about its actions. *Acidithiobacillus ferrooxidans* has been traditionally regarded as mesophilic. Recently, however, psychrotrophic strains have been isolated with a growth range on iron of 2-37 °C (Leduc and Ferroni, 1994). Some Canadian isolates have a greater cold tolerance than most strains with a temperature optimum of only 20 °C (McCready, 1988). The occurrence of broad temperature range for psychrotrophic isolates of *A. ferrooxidans* has been reported by Leduc *et al.* (1993) and by Berthelot *et al.* (1993). Temperature ranges of 2-35 °C and 4-21 °C, respectively, were observed. However, psychrophiles have not been isolated from cold tailing effluents where they would be expected (Berthelot *et al.*, 1994). The temperature used for bioleaching in most studies is 35 °C. Although *A. ferrooxidans* was reported to grow most rapidly at 30 °C, it oxidised iron faster at 35 °C (Holmes, 1988). This has important implications for industrial bioleaching since the oxidation of sulfides is exothermic, and therefore cooling may be necessary to maintain a satisfactory industrial process (Morin, 1995). Industrial applications are further described in section 8.

#### K. Metabolic pathways: involvement of *Acidithiobacillus* in bioleaching

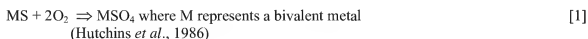
Bioleaching is a biochemical oxidation process, catalysed by a living organism, whereby an insoluble mineral is oxidised to a soluble form and recovered in a pure form. A large number of acidophilic bacteria capable of attacking mineral sulphides have been isolated from industrial leaching operations or from sites of natural leaching. *Acidithiobacillus ferrooxidans* and *A. thiooxidans* are prominent among the species isolated from leaching sites, although the redox potential and concentration of ferric iron will influence which species dominates (Rawlings *et al.*, 1999).

##### *Acidithiobacillus ferrooxidans*

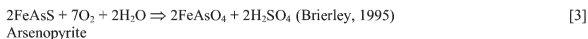
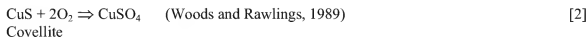
This bacterium derives its energy from oxidation-reduction reactions using insoluble sulphidic minerals as growth substrates, including pyrite ( $\text{FeS}_2$ ), chalcopyrite ( $\text{CuFeS}_2$ ), chalcocite ( $\text{Cu}_2\text{S}$ ) and sphalerite ( $\text{ZnS}$ ). This, coupled with its resistance to high concentrations of normally toxic metal ions in solution, accounts for the ubiquity of this organism in leaching systems (Cripps, 1980). Combinations of *A. ferrooxidans* and either *A. thiooxidans* or *Acidiphilium acidophilum* and *Leptospirillum ferrooxidans* have been associated with degradation of pyrite and chalcopyrite. Metals can be released from sulphidic ores by direct or indirect leaching or by galvanic conversion (Hutchins *et al.*, 1986).

*Direct leaching*

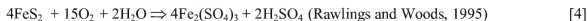
This involves oxidation of the substrate by the bacterium and may require physical attachment of the bacteria to particles of the mineral sulphide (see section 4.8). The process can be described in general by a simplified reaction:



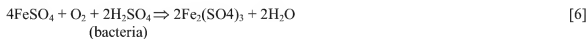
Several different metal sulphides can be acted on directly by cells of *A. thiooxidans* (Leduc and Ferroni, 1994). These include sulphides of copper, nickel, lead, iron, gallium, cobalt and zinc. Examples of direct leaching include the following:

*Indirect leaching*

This occurs through the production of an oxidative reagent or lixiviant that causes solubilisation to occur. The use of iron pyrites by *A. thiooxidans* as an energy source is a good example of how both direct and indirect leaching processes work together. The overall reaction describing pyrite oxidation is usually written as:



However, the actual pathway for the oxidation of pyrite is not a simple one-step reaction, but a series of reactions, passing through a number of intermediates. Under natural conditions, the two main oxidising agents that act on the pyrite are oxygen and the ferric ion. The reaction can be catalyzed by *A. ferrooxidans*, which increases the rate of reaction by more than  $10^6$  (Singer and Stumm, 1970).



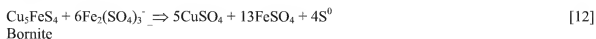
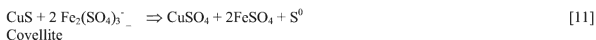
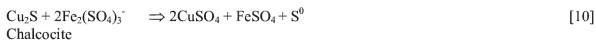
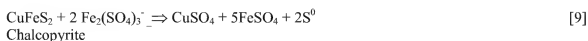
(Leduc and Ferroni, 1994; Monticello and Finnerty, 1985)

Reactions [5], [6] and [8] are part of the direct leaching mechanism, which may require the physical attachment of the bacteria to pyrite particles (section 4.8). Reaction [7] comprises the indirect mechanism that can take place independently of the bacteria. The role of the bacteria is to reoxidise the ferrous ions to ferric ions and sulphur to sulphuric acid (Monticello and Finnerty, 1985). A cyclical system develops in which ferrous ions released from pyrite are oxidised by the bacteria to ferric ion, which can then oxidise

pyrite again, generating more ferrous ions. The biomass specific oxygen consumption rate is dependent on the ratio of ferric to ferrous ions in the culture (Boon *et al.*, 1999). These ferric ions are known as the lixiviant because they carry electrons from the mineral to the bacterium's cell membrane (Leduc and Ferroni, 1994). The electrons are subsequently transported via an electron-transport chain to molecular oxygen in reaction [4]. Since iron is nearly always available in natural leaching environments, both the direct and indirect leaching mechanisms probably operate simultaneously in nature (Leduc and Ferroni, 1994; Monticello and Finnerty, 1985).

In the indirect reaction, bacterial activity is limited to the oxidation of pyrite ( $\text{FeS}_2$ ) and ferrous iron. *Acidithiobacillus ferrooxidans* does not directly interact with the metal in the minerals. The role of the bacterium is to continuously provide a powerful oxidation agent, ferric sulphate [ $\text{Fe}_2(\text{SO}_4)_3$ ] which is capable of dissolving a wide variety of metal sulphide minerals (Torma, 1991).

The following are examples of indirect leaching of minerals other than pyrites (Hutchins *et al.*, 1986)

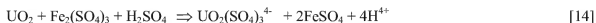


Ultimately, the indirect leaching mechanism depends on biological regeneration of ferric sulphate. Elemental sulphur ( $\text{S}^0$ ) generated by the reactions above can be converted to sulphuric acid by *A. ferrooxidans*:



The sulphuric acid maintains the pH at levels favourable to the bacteria and effectively leaches a variety of copper oxide minerals, giving copper sulphate as an end product (Hutchins *et al.*, 1986).

Uranium leaching proceeds in a similar fashion, again without the bacterium metabolising the uranium ion:



or:



*Acidithiobacillus ferrooxidans* can be used to extract uranium from sulfidic ore bodies because the bacterium uses the iron and the sulfur in the ores, as energy sources (See reaction [5]). The oxidation of reduced sulfur results in the production of sulfuric acid, whereas the oxidation of reduced iron produces the oxidant  $\text{Fe}^{3+}$ . Under such acidic conditions, the insoluble tetravalent uranium is oxidised by ferric ions to the soluble hexavalent state as follows (Berthelot *et al.*, 1993):



Thus, the soluble, oxidised uranium is released from the mineral into the leaching liquid and can be easily recovered. For other minerals where the sulphate formed from the mineral is insoluble, as is the case with lead, gold and silver, the metal may be concentrated by leaching away other soluble metal sulphates, leaving a concentrated metallic product (Hutchins *et al.*, 1986). Gold ores are often recalcitrant. The gold may be encased in a pyrite/arsenopyrite matrix that has to be decomposed before the gold is accessible to cyanide extraction (Rawlings and Woods, 1995).

#### *Galvanic conversion*

This is a lesser-known process in bioleaching where physical contact between two dissimilar metal sulphide phases immersed in an electrolyte such as dilute sulphuric acid or ferric sulphate solution creates a galvanic cell. In a mixture of pyrite and chalcopyrite, the former acts as a cathode while the chalcopyrite behaves as an anode and undergoes rapid dissolution. *Acidithiobacillus ferrooxidans* may accelerate the reaction by continuously oxidising the film of elemental sulphur that would obstruct the diffusion of copper and iron salts.

#### *Acidithiobacillus thiooxidans*

This species differs from *A. ferrooxidans* in its inability to oxidise iron ( $\text{Fe}^{2+}$ ). Sulphur appears to provide the main source of energy for this bacterium (Sokolova and Karavaiko, 1968), and its ability to oxidise elemental sulphur allows it to take part in the indirect leaching of some minerals, particularly sulphides of cobalt, nickel and zinc (Hutchins *et al.*, 1986; Norris, 1990). The sulphuric acid generated by *A. thiooxidans* through oxidation of sulphur (see reaction [8]), results in acid solubilisation of the metal. In mixed culture with iron-oxidising bacteria, *A. thiooxidans* oxidises the protective sulphur covering on the surface of minerals such as chalcopyrite. This allows bacteria such as *Leptospirillum ferrooxidans*, which cannot remove the sulphur, to attack the iron component beneath.

*Acidithiobacillus thiooxidans* can operate synergistically with *A. ferrooxidans*, or with *Leptospirillum ferrooxidans* which is able to use only ferrous iron. Either combination of bacteria can efficiently attack mineral sulphides and rapidly degrade a variety of ores (Paiment *et al.*, 2001; Rawlings and Woods, 1995). *Acidithiobacillus thiooxidans* can augment the oxidation of pyrite in coal by *A. ferrooxidans* by oxidising elemental sulphur produced by the latter organism to sulphuric acid (see reaction [3]), which is then converted by *A. ferrooxidans* firstly to ferrous sulphate and then to ferric sulphate (Ford *et al.*, 1977). Khalid and Naeveke (1991) have also observed that *A. thiooxidans* had the ability to solubilise heavy metals from carbonate-bearing complex sulphidic ore more efficiently than *A. ferrooxidans*. The reason may be that *A. ferrooxidans* alone could not produce sufficient acid to neutralise the carbonate contents and lower the pH, so that suitable conditions for growth of the bacterium could not be attained. A combination of both bacteria works efficiently since the sulphuric acid produced from sulphur by *A. thiooxidans* fulfils this condition.

### **I. Inhibition of bacterial oxidation and growth**

A number of simple organic compounds have been found to inhibit bacterial oxidation of ferrous ion at low concentrations under laboratory conditions, including benzoate, sorbate and sodium dodecyl sulphate (SDS) (Onysko *et al.*, 1984) and sodium tungsten (Sugio *et al.*, 2001). The inhibitory effect of glucose, cellobiose, galacturonic acid and citric acid compared favourably with that of SDS (Fratini *et al.*, 2000). Ferric and arsenite ions have a most detrimental effect on the growth of *A. ferrooxidans* and *A. thiooxidans* (Collinet and Morin, 1990). These compounds have been suggested for use in control of these species.

## 5. Characterisation of the Genomes (e.g. Presence of Large Plasmids, Insertion Sequences) and Stability of These Characteristics

### A. Chromosome

Recently, Rawlings (2001) provided sequencing data of the chromosome of *A. ferrooxidans* and of other iron- or sulfur-oxidising bacteria. The *A. ferrooxidans* chromosome consists of 2.9 Mb and contains 61ats larger than 500bp (Tettelin *et al.*, 2002). Harrison (1986) determined that *A. ferrooxidans* showed a wide range of genetic diversity encompassing seven DNA homology groups. This, with the discovery of high frequency mutations (Holmes *et al.*, 1988; Schrader and Holmes, 1988; Yates and Holmes, 1987), may explain the frequently observed ability of *A. ferrooxidans* to adapt to specific laboratory culture conditions such as pH and resistance to metals. High frequency mutants possess special insertion sequences that replicate and migrate along the bacterial chromosome, so that the mutants can frequently revert back to the original phenotype (Holmes and Yates, 1990). The mobility of the sequences is believed to cause a dramatic increase in the frequency of spontaneous phenotypic variations (Holmes *et al.*, 1988). Recently, this phenomenon was correlated to the high frequency of the insertion and excision of ISAfel (1.3 kb), an ISL3 family insertion sequence in a gene *ResB* that encodes for a cytochrome c-type maturation protein (Cabrejos *et al.*, 1999). In addition, Holmes *et al.*, 2001 demonstrated recently that ISAfel or similar ISAfel-1 sequences exist in diverse strains of *A. ferrooxidans* and *A. thiooxidans*.

### B. Genes

Several genes and their functions have been elucidated in the thiobacilli (Rawlings and Kusano, 1994; Rawlings and Woods, 1995; Tuovinen and Fry, 1993; Heinhorst *et al.*, 2002). A list of some of the genes in *A. ferrooxidans* have been identified and cloned as shown in Table 4.13.

Table 4.13 Genes in *Acidithiobacillus ferrooxidans*

Gene	Gene Product	Function
glnA	Glutamine synthetase	Ammonia assimilation
nifH	Nitrogenase	Reduces N <sub>2</sub> to NH <sub>3</sub>
ntrA	ntrA sigma factor	Promotes transcription
rbCL, rbcS,	RuBPCase	Fixes CO <sub>2</sub>
recA	alanyl-tRNA synthetase	Recombination/ DNA repair
merA	Mercury reductase	Resistance to Hg
merC	unnamed	Mercury transport
merR	unnamed	merA regulator
iro	Fe(II) oxidase	Iron oxidation
Rusticyanin gene	Rusticyanin	Respiratory Electron Transport Chain <sup>a</sup>
ATP synthase genes	ATP-synthase	ATP synthesis
cysC, cysD	unnamed	Sulphur assimilation

<sup>a</sup> Exact function of the gene is unknown

### C. Plasmids

Plasmids are found in a large number of *A. ferrooxidans* strains. With the exception of the arsenic and antibiotic resistance plasmids described in next section, most of the plasmids evaluated to date, are cryptic in that no phenotypic characteristic has been linked to their presence (Valenti *et al.*, 1990). Rawlings *et al.*, 1984 reported on the isolation of a 12,190 bp IncQ plasmid (pTF-FC2) from *A. ferrooxidans* present in 12-15 copies per chromosome. Recently, another IncQ plasmid pTC-F14 was isolated from *Acidithiobacillus caldus* (previously *Thiobacillus caldus*) consisting of 14 000 bp, 12-16 copies per chromosome (Gardner *et al.*, 2001). A large review of these plasmids characteristics can be found in (Rawlings and Tietze, 2001).

Dominy *et al.* (1998) isolated and characterised a 19.8 kb plasmid from *A. ferrooxidans* ATCC33020. Fourteen complete open reading frames (ORFs) were identified, most of which were proteins involved in maintenance although three of the ORFs appeared to correspond to redox-active proteins and thus could constitute part or all of an electron transport chain.

None of the plasmids describe to date, have been correlated with metal resistance (Hutchins *et al.*, 1986; Chisholm *et al.*, 1998; Leduc and Ferroni, 1993), although circumstantial evidence suggests that genes for mercury and silver resistance may be located on an unnamed  $19 \times 10^6$  daltons plasmid isolated from *A. ferrooxidans* (Visca *et al.*, 1986). One isolate of *A. ferrooxidans* was found to possess mercuric reductase activity similar to that in heterotrophic bacteria containing the mer operon, but this could not be associated with a plasmid.

Valenti *et al.* (1990) discovered a 20-kb plasmid (pTFO) in eight out of twelve strains of *A. ferrooxidans* from Italy and Mexico, which was stably maintained for many bacterial generations. All of the strains resisted similar concentrations of metal ions in spite of overall differences in their plasmid pattern.

#### **D. Genetic variation among leaching bacteria**

Suzuki *et al.* (1989) demonstrated that naturally occurring strains of *A. ferrooxidans* vary widely in their ability to utilise iron and sulphur compounds, resistance to metal and mineral leaching activities. The abilities to adsorb onto solid surfaces and to oxidise sulphur with ferric ion are other variable properties. Natural variation and /or selection of mutants can be inferred from the ease with which new isolates are selected to enhance process efficiency in various industrial settings (see section 8).

#### **E. Mutation**

The occurrence of high-frequency mutation strains of *A. ferrooxidans* was previously mentioned (section 5.1). It was noted that high frequency mutants possess special insertion sequences that replicate and migrate along the bacterial chromosome, so that the mutants can frequently revert back to the original phenotype (Holmes and Yates, 1990). The mobility of the sequences is believed to cause a dramatic increase in the frequency of spontaneous phenotypic variations (Holmes *et al.*, 1988; Holmes *et al.*, 2001). In addition, Holmes and Debus (1991) ascribed the high mutation rate of *A. ferrooxidans* to repetitive DNA sequences in the chromosomes and plasmids whose mobility around the genome could cause mutations to arise.

The high frequency of spontaneous mutations overshadows mutations derived by chemical mutagenesis and makes the latter difficult to detect.

An important implication for environmental regulation is that the inherent instability of these 'naturally' engineered strains makes it difficult to predict ecological behaviour thus complicating assessment of risk.

#### **6. Genetic Transfer Capability**

Classical genetic engineering techniques for the development of new microbial strains include mutation, conjugation, transformation, transduction and electroporation. Very little work has been done in these areas with bioleaching micro-organisms (Holmes and Debus, 1991). However, Young (1993) has presented a good review of attempts at genetic engineering of these micro-organisms, particularly *A. ferrooxidans*.

### A. Transduction

The high mutation rate of *A. ferrooxidans* was ascribed by Holmes and Debus (1991) to repetitive DNA sequences in the chromosomes and plasmids whose mobility around the genome could cause mutations to arise (see also sections 5.1 and 5.5). One of these DNA sequences has been shown to be an insertion sequence. However, bacteriophages for transduction have not been reported for *Acidithiobacillus*, although these authors cited a report of bacteriophage-like particles in acidophilic heterotrophs, which may prove valuable for developing transduction systems.

### B. Conjugation

Transfer of plasmid DNA from heterotrophic bacteria to chemolithotrophic colourless sulphur bacteria by conjugation was first achieved experimentally by Kulpa *et al.* (1983). *Halothiobacillus neapolitanus* was used in the study because of its ability to grow at the near neutral pH required by the heterotrophic donor, *Pseudomonas aeruginosa*. It was discovered that plasmid RP1, which governed resistance to three common antibiotics was accepted, replicated and expressed in the chemolithotrophic bacterium. Transfer from *Escherichia coli* into *A. thiooxidans* and back of four broad-range IncP plasmids with antibiotic resistance markers has also been achieved (Jin *et al.*, 1992), and two of the three antibiotic resistance markers have been expressed in *A. thiooxidans*.

However, although plasmids have been demonstrated in many strains of *A. ferrooxidans*, most of them are cryptic in that no identifiable phenotype has been linked to their presence (Leduc and Ferroni, 1994), and there are few cases where plasmid transfer has been successful. In spite of being cryptic in *E. coli*, however, the plasmid pTF-FC2 has been found to have a broad host-range of replication, and the discovery of such plasmids does suggest that a conjugation system exists in *A. ferrooxidans* (Rawlings and Woods, 1995; Rawlings and Tietze, 2001). This plasmid has been deemed particularly suitable for use as a cloning vector for genetic manipulation of *A. ferrooxidans* (Rawlings *et al.*, 1986). Four plasmids (pTF35, pTF-FC2, pTF3320-1, pTF3302-2) from three different strains of *A. ferrooxidans* have been successfully cloned into the plasmid pBR325 and two into the related plasmid pBR322 (Rawlings and Woods, 1995). However, the desirable properties of increased uranium and arsenic resistance, present in the *A. ferrooxidans* parents, were not expressed by the transformed *E. coli* mutants.

Two arsenic-resistant plasmids, pSDRA1 and pSDRA21 have been constructed and introduced into *A. ferrooxidans* by conjugation with *E. coli*, using Solid 2:2 Medium as a mating medium (Peng *et al.*, 1994b, 1994c). Arsenic resistance was demonstrated in the progeny. Unfortunately most attempts at returning functioning genes from *E. coli* to *A. ferrooxidans* have been unsuccessful. This means that, in principle, the statement by Holmes and Yates (1990) that genes can be extracted from *A. ferrooxidans* and be genetically modified, yet cannot be returned to *A. ferrooxidans* to create an improved organism is still largely true. However, this position may change as techniques improve for introducing foreign genetic information into *A. ferrooxidans* and other thiobacilli.

### C. Transformation

Transformation does not appear to take place naturally in *Acidithiobacillus* and other genera of colourless sulphur bacteria, but transformation has been accomplished experimentally through electroporation. Transport of naked DNA into bacterial cells by application of a high voltage electrical discharge (electroporation) has been successful in two cases. *Thiomonas intermedia* was transformed by this method using the plasmid pRK415Km (Jin *et al.*, 1994), conferring kanamycin resistance. The transformation efficiency ranged from  $10^3$  to  $10^4$  transformants  $\mu\text{g}^{-1}$  plasmid DNA under optimal conditions. Kusano *et al.* (1992) transformed *A. ferrooxidans* with natural plasmids and an artificially constructed one, but the efficiency was much less. The reason why only one strain out of the thirty tested

was amenable to transformation by electroporation is still uncertain. The plasmids in the transformed cells were stable for at least 110 generations.

## 7. Behaviour in Simulated Natural Environments Such as Microcosms

Microcosms have been used to investigate the role of *A. thiooxidans* and other thiobacilli in the degradation of concrete (Sand, 1987; Sand and Bock, 1988). Samples of concrete were inoculated in a simulation apparatus with bacteria originally isolated from concrete and incubated for nine months at an relative humidity of 95% and a temperature of 30 °C. Three compounds were tested as sources of energy: hydrogen sulphide, thiosulphate, and methyl mercaptan. Hydrogen sulphide at a concentration of 15 mg/m<sup>3</sup> resulted in severe corrosion after nine months, and *A. thiooxidans* was the dominant species in the microflora. At the lesser concentration of 2 mg/m<sup>3</sup> moderate corrosion resulted and the dominant species were *Thiomonas intermedia*, *Starkeya novella* and *Halotheiobacillus neapolitanus*. Similar results were obtained with thiosulphate but methyl mercaptan at concentrations of 22 and 2 mg/m<sup>3</sup> caused negligible corrosion and only heterotrophic bacteria and fungi thrived on the concrete blocks.

## 8. History of Use (Including Selection of Mutants and Examples of Environmental Applications of the Organism, and Information Derived from These):

### A. Selection of industrially useful mutants

The rate of growth and mineral oxidation by a population of leaching bacteria can be improved simply by cultivating a population of bacteria in a continuous flow apparatus. If the flow rate through the apparatus is slowly increased, those bacteria that are capable of the most rapid growth will replace the others. Spontaneous mutants will be selected by their growth potential on the available substrate. The advantage of this method is that it does not require sophisticated procedures, but it may take a long time to improve the bacterial strain to any economically significant extent. This approach has nevertheless been used to improve the leaching rates of *A. ferrooxidans* to several fold over that of the original isolates. Vian *et al.* (1986) were able to progressively select mutants of *A. ferrooxidans* with high oxidative efficiency at low pH values (down to pH 1.5) and with high resistance to ferric ions, thus improving the leaching of metals from low-grade ore deposits and avoiding precipitation of oxidised iron compounds.

The improvement of biomining bacteria by mutation and selection has had a dramatic effect on the economics of biooxidation of gold-bearing arsenopyrite ores in particular (Rawlings and Woods, 1995). Natural selection in the laboratory and in pilot and full-scale plants over several years has produced *A. ferrooxidans* strains that are highly resistant to arsenic and are capable of rapid oxidation of gold-bearing arsenopyrite ores in a continuous industrial bioleaching process. Highly adapted strains decompose arsenopyrite ores to an extent that allows more than 95% gold recovery in 3 days compared to the more than 12 days required by the original isolates. Further improvement however, is likely to require the application of DNA recombinant technology to amplify genes or to enable the introduction of new genetic material. Similarly, resistance to nickel ions was enhanced in *A. ferrooxidans* by repeated culturing in a medium containing nickel and gradually increasing the nickel concentration (Kai *et al.*, 1995) and the use of the modified strain, in turn, significantly increased the rate of industrial nickel extraction. No literature data was found on the selection of mutants of the three other species of *Acidithiobacillus*.

### B. Main Industrial Uses

Historically there are nine main uses to which species of *Acidithiobacillus* have been applied. This is not meant to imply that their use should be restricted to these categories, but rather to illustrate the potential and diversity of organisms within this genus. The main uses of *Acidithiobacillus* are directly related to their bioleaching ability.

**Removal of sulphides from industrial wastes**

The toxicity, corrosive properties, and unpleasant odour dictate stringent control of release of sulphides into the environment. Reduced sulphur compounds can occur in industrial wastes of the oil and gas industries as a result of several processes. Whether directly as an end product of sulphate reduction (Buisman *et al.*, 1989), or indirectly as a result of methanogenesis, the effluent from which the methane is generated may contain significant quantities of sulphate. The release of large amounts of sulphide into natural waters can result in oxygen depletion due to direct or biological oxidation (Kuenen, 1975) as well as corrosion of the concrete walls of reactors, sewer systems, and steel pipelines.

*Acidithiobacillus ferrooxidans*

*Acidithiobacillus ferrooxidans* has often been preferred for sour gas removal because the costs of neutralising sulphuric acid produced by the other bacteria can be avoided, since the sulphur is converted to ferric sulphate, and the bacterium is not inhibited by H<sub>2</sub>S (Jensen and Webb, 1995; Shiratori and Sonta, 1993). The Bio-SR Process (Sato *et al.*, 1988) comprises the following steps. Sour gas is introduced into an absorber containing ferric sulphate solution where it is oxidised to elemental sulphur and the ferric sulphate is reduced to ferrous sulphate:



Elemental sulphur is removed from the solution by a separator and the bacteria in the bioreactor then oxidise the ferrous sulphate in the solution back to ferric sulphate:



The oxidised solution is then recycled to the absorber to repeat the cycle. This procedure is environmentally sensible in that it is easy to operate, there are no waste products, no special chemicals are needed and the operating cost is low.

In earlier gas treatment processes (Onken *et al.*, 1984; Sumitomo Jukai Envirotech, 1983) the hydrogen sulphide is first precipitated as CuS or FeS. These sulphides are then oxidised by the *A. ferrooxidans* to regenerate the precipitating agent. Cadenhead and Sublette (1990) have commented that the requirement of a low pH for these processes may induce corrosion, and these authors prefer *Thiobacillus denitrificans* with its higher pH tolerance as the microbiological conversion agent.

*Acidithiobacillus thiooxidans*

Berzaczy *et al.* (1990) have patented a microbiological conversion process for degradation of sulphur-containing pollutants such as H<sub>2</sub>S, CS<sub>2</sub>, COS, thioalcohols, thioethers and thiophenes in waste gas, especially from cellulose fibre manufacture. The gas makes contact with cells of the bacterium immobilised on packing material in a packed-bed reactor. The metabolic products (mainly H<sub>2</sub>SO<sub>4</sub>) draining from the reactor are neutralised by addition of lime and lime-water.

It has been suggested that *A. thiooxidans* could be used to convert hydrogen sulphide to sulphur or sulphate in industrial plants. This process has already been demonstrated experimentally, using a continuous column contactor (Lizama and Sankey, 1993). *Acidithiobacillus thiooxidans* was also shown experimentally to act as a bacterial deodorant in removing hydrogen sulphide and trimethylamine simultaneously from a mixture of these two compounds (Hirano *et al.*, 1996). It has also been used as a deodoriser in a carrier-packed biological deodorisation reactor used in a sewage treatment plant (Shinabe *et al.*, 1995). More than 99% of the hydrogen sulphide and 70-80% of the methanethiol were removed from

the raw gas in the early section of the packed bed. Lizama and Sankey (1993) pointed out that *A. thiooxidans* might have some advantages over *T. denitrificans*, since the energy requirement of *A. thiooxidans* for fixing carbon dioxide from the atmosphere is high. It must oxidise large quantities of sulphide for the production of relatively little biomass, and its tolerance to low pH makes it resistant to the sulphuric acid produced.

### ***Removal of heavy metals from sludge and mine wastes***

#### *Extraction of heavy metals from sewage sludge*

Application of sewage sludge to agricultural land is one of the most economical methods for final sludge disposal (Bruce and Davis, 1989), since it is a very good soil conditioner and sources of plant nutrients. However, the levels of toxic metals in sewage sludge make them unsuitable for agricultural land application because food plants for humans and animals take up these metals, causing them to accumulate in the food chain (Tyagi *et al.*, 1993b).

Couillard and Mercier (1994) determined that bacterial leaching of metals from sludge using *A. ferrooxidans* was more economical than traditional methods of sludge management except in the case of a processing plant treating only 20,000 m<sup>3</sup> of wastewater per day. Furthermore, the use of biological leaching had less of an environmental impact and the product was acceptable for use on agricultural lands (Wong and Henry, 1984). Bacterial leaching was also more economical because acid consumption is reduced by more than 80% (Couillard and Mercier, 1991).

The presence of sulphur-oxidising microflora in sewage sludge is potentially useful for the removal of toxic metals found there (Blais *et al.*, 1993). An acid medium is useful in inactivating many bacteria and viruses, though not all organisms are affected. The process is enhanced through the addition of elemental sulphur as an energy substrate, preferably in solid rather than powdered form since the sulphur is then easier to recover at the end of the operation (Ravishankar *et al.*, 1994). Rapid decrease of sludge pH by a mixed culture through sulphur oxidation into sulphuric acid solubilised toxic metals to levels recommended for intensive use of residual sludge in agriculture (Tyagi *et al.*, 1993b). The solubilised metals in the leachate could be separated from decontaminated sludge solids by centrifugation or filtration, precipitated by neutralising the leachate with lime and then safely disposed of. There exists the potential to recycle these metals for the metal industry. Decontaminated sludge solids must also be treated with lime to reduce acidity before application to agricultural land (Tyagi *et al.*, 1993b).

Evidence seems to indicate that a combination of thiobacilli is more effective in the treatment of sewage sludge than the use of a single species. *Acidithiobacillus ferrooxidans* on its own, was not as capable of efficient solubilisation of metals as when compared to a mixture of indigenous sulphur-oxidising bacilli or with other *Acidithiobacillus* species (Couillard and Zhu, 1992; Tyagi and Couillard, 1987; Tyagi *et al.*, 1993b). In Table 4.14, the efficiency of thiobacilli species to solubilise heavy metals is illustrated.

**Table 4.14 Comparative % solubilisation of heavy metals in sludge**

Heavy Metals	Indigenous bacterial microflora Wong and Henry (1984)	<i>A. ferro-oxidans</i> Tyagi <i>et al.</i> (1993a)	<i>A. ferro-oxidans</i> + <i>A. thiooxidans</i> Tyagi and Couillard (1987)	Mixture of thiobacilli* Tyagi <i>et al.</i> (1993b)	<i>T. thio-parus</i> + <i>A. thio-oxidans</i> Blais <i>et al.</i> (1992)	<i>T. thio-parus</i> + <i>A. thio-oxidans</i> Blais <i>et al.</i> (1993)
Cd	80-85%	55-98%	50%	51-93%	83-96%	83-90%
Cr	-----	0-32	-----	16-58	16-54	19-41
Cu	66-80	39-94	75	47-95	85-87	69-92
Mn	70-78	71-98	-----	-----	91-94	88-99
Ni	37-98	37-98	-----	48-97	78-79	77-88
Pb	0	0-31	55	7-63	28-46	10-54
Zn	84-90	66-98	96	65-98	82-96	88-97

\*Named species were *A. ferrooxidans* and *A. thiooxidans*. Other sulphur oxidising organisms were also present (e.g. *Sulfolobus acidocaldarius*)

Blais *et al.* (1992, 1993) showed that the bioleaching of metals from sewage sludge could be carried out by successive growth of moderately acidophilic bacteria (*H. neapolitanus*, *T. denitrificans*, *T. thio-parus*) and the acidophilic *A. thiooxidans*. *Thiobacillus thio-parus* VA-7 and *A. thiooxidans* VA-4 possess distinctive physiological characteristics that allow them to easily grow and solubilise heavy metals in municipal sludge (Blais *et al.*, 1992). Strain VA-7 decreased the pH of the sludge initially from pH 7-8.5 to a value between pH 4.0 and 4.5. Strain VA-4 began to grow and further reduced the pH to values below 2.0.

#### *Extraction of heavy metals from industrial wastes*

Bosecker (1987) found that some products such as copper, chromium, zinc and vanadium were completely extracted from a variety of industrial waste products by the sulphuric acid produced by *A. thiooxidans*. In some cases bacterial leaching was as effective as chemical leaching with sulphuric acid. Heavy metals such as Cu, Pb, Zn, Fe, As and Cd could also be recovered from flue dust from a flash-smelting furnace (Shiratori and Sonta, 1993) using *A. ferrooxidans*. There were several advantages to bacterial oxidation, including low cost and clear separation of metals.

Aluminium could be recovered from red mud, a chemical waste produced by alkaline extraction of aluminium from bauxite (Bayer process). The mud usually contains about 25%  $Al_2O_3$  (Vachon *et al.*, 1994) and is still highly alkaline (pH 12-13). Traditionally, red mud has either been disposed of in the sea or allowed to settle in 'red lakes' for further processing; neither process is environmentally innocuous. Bioleaching, after the addition of sewage sludge to a concentration of 30% V/V to red mud, solubilised up to 47% of the aluminium and brought down the pH to 3.5-2.2. The bacteria responsible were not identified but were probably *T. thio-parus* and *A. thiooxidans*.

Silver recovery from waste photographic processing solutions has been accomplished using *A. ferrooxidans*, *A. thiooxidans*, *Starkeya novella*, *T. denitrificans* and *T. thio-parus* (Kitajima and Abe, 1979).

#### *Biomining and acid production*

Bacterial leaching is used in the recovery of metals from ores that are often too poor for conventional metallurgical extraction methods (Robertson and Kuenen, 1992; Paiment *et al.*, 2001). The main metals that have been recovered on a commercial scale by microbial leaching are copper and uranium. In the U.S.A., 10-15% of all copper is obtained in this way (Cripps, 1980). The potential of this technique, however, is not limited to copper and uranium, since it can, in principle, be extended to all sulphide and

some oxide ores. Other metals that have been extracted using processes that involve bacteria include zinc, cobalt, lead, gold and molybdenum (Robertson and Kuenen, 1992).

Biological copper leaching is practised in many countries, including the U.S.A., Russia, Chile, Peru, Australia, Spain, Canada and Mexico. Typically, copper ore mined from open pits is segregated, higher-grade material being concentrated for smelting and the lower-grade ore subjected to leaching. The latter is piled to form a 'dump' up to 40 m high and several hectares wide. After the top is levelled, a leaching solution containing ferric sulphate and *A. thiooxidans* is flooded or sprayed onto the dump (Merson, 1992). Bacterial colonisation occurs mainly in the top metre. Leachates enriched with copper exit at the base of the dump and are conveyed to a central recovery facility. The copper in the solution is recovered by mixing it with iron scraps in large container units according to the following reaction:



The finely divided "cement copper" is periodically recovered and refined for sale, while the barren solution is recycled to the leachate dumps. A typical large dump may have an operating life of over ten years (Hutchins *et al.*, 1986). Total copper recoveries of 80% were attained by the Chilean company *Sociedad Minera Pudahuel* (SMP) after leaching times ranging from 150 to 230 days, and about 90% recovery was attained after 7 to 11 months, more than double the quantities that would have been obtained without bacteria (Acevedo and Gentina, 1993).

*Acidithiobacillus thiooxidans* has also been used to obtain a high degree of copper extraction from covellite (Curutchet *et al.*, 1995) by oxidising the layer of sulphur covering the sulphide surface and allowing sulphide oxidation by ferric ion. Both *A. thiooxidans* and *A. ferrooxidans* are effective in leaching covellite, although at different rates (Donati *et al.*, 1996).

*Acidithiobacillus ferrooxidans* was used to extract uranium from low-grade ores, as for example in the Denison Mine project in Elliott Lake, Ontario, Canada (Brierley, 1990; McCready, 1988). The operation is similar to copper leaching in that it employs a bacterially assisted, flood-leaching process, but it is performed underground on mine waste rubble. About 12-13% of the uranium production is attributed to bioleaching at present, which could increase to 25% after refinement. After the uranium-bearing ore is leached, a uranium-bearing solution drains to lower portions of the mine and accumulates in sumps, after which it can be pumped to the surface for uranium recovery (Hutchins *et al.*, 1986). The addition of elemental sulphur or sulphur slag as an external energy source enhanced the leaching process (Bhatti *et al.*, 1991).

*Acidithiobacillus ferrooxidans* and *A. thiooxidans* were used together to extract cobalt from an ore containing 40.3% iron and 1.4% cobalt (Battaglia *et al.*, 1994). It was found that for the system to operate at the highest efficiency, the acidity had to be maintained at a pH of between 1.1 and 2. Furthermore, the dissolution of pyrite was depressed when the concentration of ferric ions reached a level of 35 g/L.

According to Brierley (1995), an estimated one-third of the world's total gold production is now from refractory deposits such as gold-arsenic concentrates. In these concentrates, gold and silver are finely disseminated in sulphide minerals, mainly arsenopyrite as well as pyrite, and partly antimonite (Karavaiko *et al.*, 1986). Bio-oxidation, in which chemolithotrophic bacteria such as *A. ferrooxidans* have been used to decompose the ore, is a low-energy alternative to conventional methods which involve roasting and pressure leaching.

Lindstrom *et al.* (1992) and Morin (1995) have reviewed the process of microbial leaching for recovery of gold. *Acidithiobacillus ferrooxidans* was found to rapidly and selectively oxidise arsenopyrite and other sulphide minerals in the concentrates. Bio-oxidation increased gold recovery from low levels up

to 95-99% (Maturana *et al.*, 1993; Morin, 1995) in comparison with traditional acid leaching. Compared to roasting, bio-oxidation with thiobacilli and their relatives could generally reduce capital costs by 12-20%, operating costs by 10% in some cases and construction time by 25% (Brierley, 1995). At the same time, the process was less polluting to the environment and had lower energy requirements since it operated at relatively low temperatures (Cripps, 1980). Highly adapted strains decompose arsenopyrite ores to an extent that allows more than 95% gold recovery in 3 days compared to the more than 12 days required by the original isolates.

*Acidithiobacillus ferrooxidans* accelerated the leaching of silver and other metals present in a mixed sulphide ore from Idaho (Ehrlich, 1986; 1988). Continuous leaching where iron in the solution was supplied to the reactor from a reservoir resulted in selective leaching of the silver.

Zinc has been effectively recovered from a zinc sulphide concentrate by continuous microbiological leaching with *A. ferrooxidans* using a two-stage reactor sequence (Sanmugasundaram *et al.*, 1986). Direct leaching by *A. ferrooxidans* together with *A. thiooxidans* has been found to be effective (Pistorio *et al.*, 1994).

Sub-marginal mercury/antimony sulphidic ores were separated under experimental conditions into their components using a culture of *A. ferrooxidans* isolated from a coal field in the Moscow region (Lyalikova and Lyubavina, 1986).

#### ***Oil recovery and purification of oil shale***

The position with oil reserves is similar to that of metallic ores outlined above: some 30,000 billion barrels of oil are present in shales, of which only about 2% are available because the recovery of the rest is uneconomical. Conventional extraction methods involve crushing and heating the shale to high temperatures to release the oil from the inorganic matrix. In this way, vast quantities of energy are consumed, only 75% of the organic material is liberated and large quantities of expended shale must be disposed of (Dalton, 1979). A biological process has been invented (Yen *et al.*, 1976) to extract oil at ambient temperatures, giving good yields and avoiding the production of vast quantities of insoluble residue. Organisms mentioned in the patent included *A. ferrooxidans*, *A. thiooxidans*, *H. neapolitanus*, *T. thioparus*, other species of *Thiobacillus* since relocated, and various species of *Desulfovibrio*. These bacteria remove most of the organically bonded disulphides and polysulphides in the inorganic matrix of the shale oil, leaving an organic structure that can be used as fuel or can be converted into other materials such as petroleum or synthetic natural gas.

Bioleaching of pyrite from the Aleksinac oil shale in Yugoslavia was successfully carried out by using *A. ferrooxidans* as an alternative to chemical removal; chemical removal formerly led to undesirable changes in the oil substrate (Vrvic *et al.*, 1988).

#### ***Desulphurisation of coal***

To some extent coal desulphurisation is similar to the process above, in that in both cases sulphidic ores are oxidised; however both the aim and the end products are different. Coal, being of fossil origin, is not a homogeneous substance, containing a variable quantity of fixed carbon, hydrogen, oxygen, sulphur, nitrogen and trace minerals (Mannivannan *et al.*, 1994). The aim of coal desulphurisation is to produce coal which is as free of sulphur and its derivatives as possible and it is necessary therefore to convert reduced sulphur compounds to soluble forms (Robertson and Kuenen, 1992).

Microbial desulphurisation by *A. ferrooxidans*, *A. thiooxidans* and *Sulfolobus brierleyi* can remove 90% or more of the inorganic sulphur from coal within a few days (Khalid and Aleem, 1991; Kilbane, 1989). The pyrite-oxidising capacity of *A. ferrooxidans* and related organisms has also been successfully

exploited in the desulphurisation of coal (Bos *et al.*, 1988; Bos and Kuenen, 1990; Tuovinen and Fry, 1993) with the production of sulphuric acid instead of sulphur dioxide.

Sulphur is bound in inorganic and organic form in coal. Sulphur dioxide emissions arising by coal burning represent an important ecological problem, which can be solved by the conversion of the sulphur compounds in the coal into different end products (Beck *et al.*, 1988). These workers found that *A. ferrooxidans* was the most useful species to use, but other species such as *A. acidophilum*, (*T. acidiphilus*), *Thiomonas perometabolis* (*T. perometabolis*) and *T. plumbophilus* could also be used. Beyer *et al.* (1988) and Bos *et al.* (1988) found that it was possible to remove 90% of the pyrite from coal within 8 to 10 days, using a mesophilic pyrite-oxidising microbial system for which a plant design involving a cascade of Pachuca tank reactors was devised.

Mixed cultures of *A. ferrooxidans* and *A. thiooxidans* have also been used to remove sulphur from lignite, the lowest rank of coal intermediate between peat and anthracite (Raman *et al.*, 1994).

#### ***Desulphurisation of rubber***

Mixed cultures of *A. ferrooxidans* and *A. thiooxidans* satisfactorily removed sulphur inclusions in rubber materials that could be recycled from urban wastes (Torma and Raghaven, 1990). The two bacteria together were more efficient than the individual bacteria alone.

#### ***Detection of sulphur impurities in wine***

A rapid and accurate sensor system was developed to determine free sulphite in wine (Nakamura *et al.*, 1989), using immobilised cells of *A. thiooxidans* S3. The concentration of free sulphite could then be controlled so as to protect wine from oxidation processes and microbial spoilage. The same strain was used to detect sulphur dioxide in wine and various foodstuffs. The bacterium is converted into a microbial sensor by setting a piece of microbial membrane onto an O<sub>2</sub> electrode soaked in 0.1 M citrate buffer and covered with a gas-permeable Teflon membrane (Kawamura *et al.*, 1992; Kurosawa *et al.*, 1990, 1994). A similar biosensor using strain JCM7814 was developed to detect concentrations of sulphur dioxide in wine up to 50 mg/l, with a limit of detection of 5 mg/l and a response time of 20 minutes (Nakamura *et al.*, 1993).

#### ***Agricultural fertilisation***

Since thiobacilli are involved in the sulphur cycle, the presence in the soil can be used to assess fertility. In Australia, for example, thiobacilli are scarce in sulphur-deficient areas (Kuenen *et al.*, 1992).

Under warm climatic conditions rock phosphate pelleted with sulphur and seeded with thiobacilli has been shown to be a useful slow release source of phosphate and sulphate for soil fertilisation (Swaby, 1975). However, the addition of *A. thiooxidans* to a mixture of rock phosphate and sulphur granules, called 'Biosuper', has given variable results; in some cases the mixture increased the level of phosphorus in the soil and gave plant yields equivalent to those produced by the more expensive super-phosphate (Muchovej *et al.*, 1989; Schofield *et al.*, 1981), whereas in others, addition of *A. thiooxidans* did not improve performance of the fertiliser (Alvarez *et al.*, 1981; Rajan, 1982). In pot experiments with guavas and ryegrass, the presence of *A. thiooxidans* in the soil enhanced the uptake of Fe, Zn and Mn (Azzazy *et al.*, 1994; Schnug and Eckardt, 1981).

*Acidithiobacillus thiooxidans* is perhaps more useful in low-cost production of a supply of sulphuric acid for the dissolution of apatite in the production of phosphate fertilisers (Donati and Curutchet, 1995).

### ***Soil Reclamation***

Incorporating certain species of *Thiobacillus* with sulphur enrichment can reclaim alkali soils, since these soils are naturally poor in sulphur-oxidising bacteria. *Thiobacillus thioparus* has been inoculated with crushed sulphur into a calcareous solonchic soil in virgin Alberta prairie land in order to promote acidification. This, combined with ripping the soil at a 60 cm depth and weekly irrigation, released several soluble salts, particularly those of sodium, calcium and magnesium (Bole, 1986). *Acidithiobacillus thiooxidans* was used to reclaim a saline alkaline soil by inoculating it together with elemental sulphur, thus lowering the pH and increasing the quantity of soluble salts (Bardiya *et al.*, 1972).

## **III. Human Health Considerations**

### **1. Diseases Caused and Mechanism of Pathogenicity, Including Invasiveness and Virulence**

No species of *Acidithiobacillus* are known to be pathogenic based on the results of literature search conducted in various databases such as PubMed, Biosis, CAB Health and Current Contents.

### **2. Toxicogenicity**

There is no evidence to indicate that any species of *Acidithiobacillus* are toxigenic based on the results of literature search conducted in various databases such as PubMed, Biosis, CAB Health, Toxnet and Current Contents.

### **3. Allergenicity**

No literature was found on the allergenicity of thiobacilli. However, the organisms are Gram negative and would therefore be expected to exhibit some of the characteristics associated with endotoxin. Nevertheless, no allergens of significance to humans have as yet been traced to this group of bacteria.

## **IV. Environmental and Agricultural Considerations**

### **1. Natural Habitat and Geographic Distribution: Climatic Characteristics of Original Habitats**

#### **A. General overview**

Although *Acidithiobacillus* is probably widely distributed, this distribution is usually related to the presence of sulphur as for example, coastal ecosystems such as salt marshes, sediments, mine dumps and sulphur-rich products of human industrial activity such as metal pipelines and concrete (Kelly and Harrison, 1989; Smith and Strohl, 1991).

As well, some of the products of human industrial activity such as metal pipelines and concrete have become a new ecological niche for some species. This has potentially destructive consequences (see below).

#### **B. Correlation of natural incidence with usage and environmental impacts**

While it can be assumed that *Acidithiobacillus* plays a role in the sulphur cycle, the extent of their involvement is largely unknown. Vitols and Swaby (1969) showed that most thiobacilli in Australian soils rich in sulphur were autotrophic. *Acidithiobacillus* species' natural habitats, usage and environmental impacts are presented in Table 4.15.

Table 4.15 Usage and environmental impacts of *Acidithiobacillus*

Species	Physiological status	Natural habitats	Usage	Impacts
<i>Acidithiobacillus albertensis</i>	AA	Acid sulphurised soil (Bryant <i>et al.</i> , 1983)	None at present	Not known
<i>Acidithiobacillus caldus</i>	AF	Coal spoil (Hallberg and Lindstrom, 1994)	None at present	Not known
<i>Acidithiobacillus ferrooxidans</i>	AA	Sulphurised soil and rock, in nature, e.g. pyrite (FeS <sub>2</sub> ). Iron and sulphur springs, sulphur iron-rich acidic waters, mines with various ores (Berthelot <i>et al.</i> , 1993; Blowes <i>et al.</i> , 1995; De Kimpe and Miles, 1992; Harrison, 1982; Johnson, 1995b; Valenti <i>et al.</i> , 1990; Vitolins and Swaby, 1969; Zagury <i>et al.</i> , 1994).	<ul style="list-style-type: none"> <li>•Removal of heavy metals,</li> <li>•Bioleaching of ores, Desulphurisation of coal and rubber.</li> </ul>	Pyrite-oxidising bacterium involved in Acid Mine Drainage (AMD) (Evangelou, 1995)
<i>Acidithiobacillus thiooxidans</i>	AA	Sulphurised soil and deposits fresh water, mines + various ores, corroded concrete (Cho and Mori, 1995; Emde <i>et al.</i> , 1992; Evangelou and Zang, 1995; Fliermans and Brock, 1972; Harrison, 1982; Parker, 1945; Robertson and Kuene, 1992; Sokolova and Karavaiko, 1968).	<ul style="list-style-type: none"> <li>•Oxidation and removal of sulphidic pollutants in gas</li> <li>•Recovery of heavy metals</li> <li>•Recovery of silver in photoprocessing,</li> <li>•Recovery of certain ores</li> <li>•Sulphur transformation</li> <li>•Desulphurisation of coal and rubber</li> <li>•Detection of sulphur impurities in wines</li> <li>•Enhancement of phosphorus fertiliser</li> <li>•Soil Amelioration</li> </ul>	<ul style="list-style-type: none"> <li>•Potential threat to buildings, drains,</li> <li>•Deterioration of rubber,</li> <li>•Pyrite-oxidising bacterium involved in AMD (Evangelou, 1995),</li> <li>•Development of acid soils (Arkesteyn, 1980).</li> </ul>

AA = Acidophiles, strictly chemolithotrophic and autotrophic

AF = Acidophiles, facultatively chemolithotrophic or mixotrophic

It is assumed that all thiobacilli play some role in the sulphur cycle.

## 2. Significant Involvement in Environmental Processes, Including Biogeochemical Cycles and Potential for Production of Toxic Metabolites

### A. Utilisation of sulphur

#### *The sulphur cycle*

Colourless sulphur bacteria, which include the thiobacilli, play an important role in the sulphur cycle by oxidising sulphur and sulphides to sulphates so that they can be utilised by plants (Weir, 1975). Sulphide, which originates from anaerobic sulphate reduction and from decaying organic matter, is

oxidised to sulphate under both aerobic and anaerobic conditions and by both chemical and biological means. Sulphate is assimilated by plants and micro-organisms and reduced to sulphides by other micro-organisms when these die.

In nature, a variety of reduced inorganic sulphur compounds occur as intermediates between sulphide and sulphate, which normally react very slowly with oxygen. Biological oxidation by the colourless sulphur bacteria plays an important role in the recycling of reduced sulphur compounds under aerobic conditions (Kuenen, 1975). The thiobacilli have received more attention than the other main groups of sulphur-oxidising micro-organisms. These comprise several genera of heterotrophic and facultative autotrophic bacteria and yeasts and are far more numerous than the thiobacilli. The thiobacilli, however, were deemed to be more efficient when conditions suited them (Vitolins and Swaby, 1969).

### *Role of thiobacilli in geologic sulphur deposits*

Evidence shows that thiobacilli play a fundamental role in the development and weathering of sulphur deposits. In a review of sulphur deposits and waters with high sulphidic content in the former USSR, Sokolova and Karavaiko (1968) found that *T. thio-parus* and *A. thiooxidans* were often associated. However, slight differences in distribution, according to the redox potential and the acidity of the environment, roughly correlating to the pH ranges cited in Table 4.12 were observed. Two examples show that these bacteria may play a role in the build-up and breakdown of sulphur deposits:

#### *Formation of sulphur deposits*

In the Shor-Su sulphur mines, *A. thiooxidans* occurred in the upper horizons of the deposit where an oxidative environment prevailed and the rocks were highly acidic (Sokolova and Karavaiko, 1968). In the aquifers throughout the lower horizon where the pH was neutral or weakly alkaline due to the proximity of limestone, *T. thio-parus* was widespread and *A. thiooxidans* was absent. The presence of hydrogen sulphide prevented oxidation of the ore bed in the main deposit. Hydrogen sulphide was produced daily up to a rate of 0.2 mg/l by numerous sulphur-reducing bacteria in the groundwater and in the rocks, and ascended towards the surface waters where it was oxidised by *T. thio-parus* to sulphur and water.



In several parts of the sulphur mines, sedimentation of molecular sulphur still continued by oxidation of the hydrogen sulphide of the underground waters, as shown by the presence of bacterial cells and small, freshly-deposited sulphur crystals retrieved on culture slides.

#### *Degradation of sulphur deposits in the soil*

In the early 1970s, millions of tonnes of elemental sulphur were extracted from sour natural gas and stored in blocks in Alberta, Canada (Maynard *et al.*, 1986). Since 1979, large quantities of sulphur have been deposited in adjacent forest systems due to mechanical break-up and weathering of these blocks, causing considerable damage to the understory vegetation. Three sites at distances of 50, 250 and 750 m from a sulphur block were studied. The pH was 2.6, 3.7 and 4.4 respectively for each site. This increasing acidification with the declining proximity to the sulphur block, was attributed to *A. thiooxidans*, which was the main soil micro-organism responsible for elemental sulphur oxidation at all three sites. *Thiobacillus thio-parus* was also present, but at a significantly lower population level at the first site (that with the lowest pH) than at the other two. The nutrient concentration of the soil as measured by recoverable calcium, magnesium and potassium also decreased sharply towards the sulphur block.

### ***Reaction on pyrite (FeS<sub>2</sub>) in nature***

Pyrite is the most prevalent form of iron disulphide and is usually associated with coalfields in the U.S. and elsewhere in the world. It is associated with many ores, including zinc, copper, uranium, gold and silver. Pyrite is formed in a reducing environment with a continuous supply of sulphates and iron in the presence of easily decomposable organic matter (Evangelou and Zhang, 1995).

Oxidation of pyrite deposits due to the combined action of *A. ferrooxidans* and *A. thiooxidans* in empoldered or flooded land has caused a pronounced acidification of the soil (Arkesteyn, 1980; Kuenen, 1975). When soils rich in pyrite are brought into agricultural production, “cat clay” is often formed where clay particles are cemented together by jarosite formed during the oxidation of pyrite, reducing agricultural production (Kuenen, 1975; Pronk and Johnson, 1992). Jarosite [KFe<sub>3</sub>(SO<sub>4</sub>)<sub>2</sub>(OH)<sub>6</sub>] is a basic ferric sulphate also found in deposits associated with pyrite (Ivarson, 1973). *Acidithiobacillus ferrooxidans* has been shown to play a part in the weathering of sulphide minerals with jarosite formation under humid conditions in metamorphic and igneous rocks in Ontario (De Kimpe and Miles, 1992). Other acidophilic species, such as *T. prosperus*, are able to perform the same reactions (Johnson, 1995b) but strains of *A. ferrooxidans* tend to grow more rapidly on ferrous iron than do other iron-oxidising acidophiles, thus causing them to dominate mixed populations (Pronk and Johnson, 1992).

### ***Acid mine drainage (AMD)***

Acid and metal pollution can be the result of the activities of thiobacilli in mine wastes (Tuovinen and Kelly, 1972). The natural oxidation of sulphide to sulphates, including sulphuric acid, as part of the sulphur cycle has become greatly enhanced by the world's increasing demand for metals and fossil fuels. Acid mine drainage (AMD) result from land disturbances due to mining and ore processing, and has become an economic and environmental burden (Evangelou and Zhang, 1995). Acid mine drainage may be enriched with soluble iron, manganese, aluminium, sulphate and heavy metals, and the pH may be as low as 2. One of the effects of AMD is to kill established vegetation associated with mine sites and spoil tips. The typical situation found on a reclaimed spoil tip is a slow fall in pH over most of the site. Severe acid generation tends to neutralise any added lime or buffering systems in the soil quickly, and further accelerates the rate of oxidation of pyrite (Backes *et al.*, 1986). The large quantities of sulphuric acid that are produced make the environment in which *A. ferrooxidans* grows, and to which it is well adapted, inhospitable to most other organisms (Rawlings and Woods, 1995; Léveillé *et al.*, 2001; Leduc *et al.*, 2002).

Mine spoils that were alkaline in nature (pH 9), with low sulphur content and a high concentration of chlorides tended to be free of *A. ferrooxidans* (Twardowska, 1986). The limiting pH value for growth of *A. ferrooxidans* in rock material and drainage was found to be about 7.2 (Twardowska, 1987).

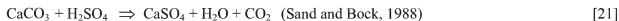
## **B. Corrosion**

Because many of the colourless sulphur bacteria produce sulphuric acid or ferric ion, they are often associated with the oxidative corrosion of concrete and pipes, and have been implicated in the corrosion of buildings and ancient monuments.

### ***Corrosion of concrete by Acidithiobacillus thiooxidans (Thiobacillus concretivorus)***

Bacterial involvement in concrete corrosion has been known since the pioneering work of Parker (1945). *Acidithiobacillus thiooxidans* has been found mainly responsible for the deterioration of concrete in sewage pipelines (Cho and Mori, 1995; Jozsa *et al.*, 1995; Mori *et al.*, 1991, 1992; Parker, 1945; Sand and Bock, 1988, 1991). The bacterium is able to use hydrogen sulphide released from the sewage and oxidise it to sulphuric acid which then attacks the concrete. It was found to coexist with an acid-resistant

fungus which could oxidise  $\text{H}_2\text{S}$  to thiosulphate, and it is thought that the bacterium used the latter as an energy source, producing sulphuric acid that was responsible for the corrosion.



The calcium loss results in a reduction in the stability of the structure.

The pH of fresh concrete sewer pipes is about 13, so that little microbial activity would normally occur in them. The pH would be lowered by carbonation of the concrete due to exposure to air, as well as by exposure to  $\text{H}_2\text{S}$  and then by the activities of the bacterium itself. Other bacteria, including *H. neapolitanus*, *T. intermedia* and *S. novella*, have been found to accompany *A. thiooxidans* but played a minor role in corrosion (Sand and Bock, 1991).

*Acidithiobacillus thiooxidans* was shown experimentally to be able to degrade Sulphlex, a mixture of elemental sulphur and plasticisers used as a paving material and as a substitute for asphalt in road construction with a simultaneous production of sulphuric acid (Ferenbaugh *et al.*, 1992). In concurrent studies, plants grown in soils amended with Sulphlex exhibited higher sulphur content and reduced growth consistent with poisoning. Indications are, therefore, that *A. thiooxidans* has the potential for adverse effects on sulphur-containing construction materials as well as on the local environment.

### ***Corrosion of steel***

Transportation of low-grade coal in railway carriages has been linked with accelerated corrosion of the steel framework (Brozel *et al.*, 1995), involving scaling, pitting and cracking. Corrosion of 3CR12 steel coupons embedded in coal occurred under experimental conditions after the pH of the coal had been lowered to 2.5. The extent of the damage increased when the fungus *Hormoconis resinae*, another dominant member of the natural flora, was present in the substrate.

### **C. Deterioration of rubber**

Thaysen *et al.* (1945) reported that the deterioration of rubber hoses was the result of the microbial oxidation of elemental sulphur present in the rubber by *A. thiooxidans*. This was confirmed by Raghaven *et al.* (1990) who also note similar effects on polyethylene.

## **3. Interactions with and Effects on Other Organisms in the Environment**

*Acidithiobacillus* plays an important role in making sulphur available to plants. *Acidithiobacillus thiooxidans* may also make phosphorus, iron, zinc and manganese more available for plant growth. However, plants can be adversely affected by too high concentrations of sulphate and increased acidity in the soil as a result of activity due to *A. thiooxidans*. Very little is known about antagonists of these bacteria.

## **4. Routes of Dissemination: Biological or Physical**

### **A. Biological**

All species of *Acidithiobacillus* are motile (Kelly and Wood, 2000) so that they are able to disseminate within their immediate environment.

### **B. Physical**

Water appears to be the major means of passive dissemination, and some dispersal must also be due to the spread of particles of soil or rock to which the bacteria have become attached.

## V. Application of the Organism in Industry

### 1. Containment and Decontamination

#### A. Chemical Methods

The traditional method for controlling acidity in coal spoil and the deposition of pyrite in field drains and soils was to add high levels of lime in order to maintain the pH well above 4 to limit the activity of ferric ions and of *A. ferrooxidans*, so restricting the oxidation of pyrite to a process involving oxygen alone (Backes *et al.*, 1986; Poissant, 1986; Trafford *et al.*, 1973). Alkaline chemicals, such as limestone, sodium carbonate or sodium hydroxide, have been applied or pumped into active mines to neutralise acid soils. Limestone could hydrolyse most heavy metals, precipitating them as metal hydroxides (Evangelou and Zhang, 1995). This method depended on maintenance of the pH at a consistent value, and very often reacidification occurred as the lime was neutralised or washed out of the surface layers. Limestone is usually readily available but massive over liming of the site may result (Pulford *et al.*, 1986) and its effectiveness is reduced because a coating of ferric hydroxide precipitates develops to shield it from further dissolution (Evangelou and Zhang, 1995). The use of soluble neutralising agents such as sodium hydroxide avoids this problem but can be costly and not very practical.

McCready (1987) successfully controlled *A. ferrooxidans* in pyritic shale in the laboratory by adding sodium chloride to reach a concentration of 1.5%. He has suggested that the incorporation of a salt layer would prevent AMD in pyritic sites. Another promising method of controlling pyrite oxidation is considered to be the application of phosphate, which can precipitate  $\text{Fe}^{3+}$  in an insoluble form as  $\text{FePO}_4$  or  $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$  (strengite). Coating of the pyrite with iron is prevented by leaching the pyrite with low but critical concentrations of  $\text{H}_2\text{O}_2$  and a pH buffer, with or without  $\text{KH}_2\text{PO}_4$ . In the first case, iron phosphate precipitates as a coating on the pyrite surface; in the second, it precipitates as an iron oxide (Evangelou and Zhang, 1995). Treatments with phosphate, silicate, citrate and EDDHA (ethylenediamine di-orthohydroxyphenylacetic acid) inhibited the release of acid and iron from pyritic mine waste (Pulford *et al.*, 1986).

Growth of *A. thiooxidans* strain NBI-3 was also greatly reduced in the laboratory by 5 mM of  $\text{NiSO}_4$ , the nickel binding to the cells and inhibiting the enzymes involved in sulphur oxidation (Maeda *et al.*, 1996).

Inhibition of iron-oxidising bacteria may be achieved through the use of anionic surfactants (including common cleaning detergents), organic acids and food preservatives (Kleinman, 1989). Acid production may be reduced by 60 to 95%. However, wide use of surfactants is limited by the necessity for frequent treatments, since they are very soluble and motile, and they may also be adsorbed onto the surfaces of minerals without reaching the pyrite-bacterial interface (Evangelou and Zhang, 1995). The Witco product Microwet II<sup>TM</sup> incorporating various surfactants, when continuously applied to refuse leaving a coal mine satisfactorily controlled *A. ferrooxidans* and reduced acid production (Stancel, 1982). Sodium lauryl sulphate was shown to be inhibitory at a concentration of about  $10^{-6}$  M in culture media and in mine spoils at higher concentration (Fox and Rastogi, 1983). It also has the additional benefit of being low in mammalian toxicity and quite biodegradable, so subsequent environmental problems are unlikely. For restoration of a good ground cover over reclaimed overburden, these authors proposed that a controlled release system be set up to promote a good ground cover, which, over a period of years, would eventually deprive the mine spoil of oxygen and water needed to generate acidity. 0.25% Sodium dodecyl sulphate at about 5,000 litre ha<sup>-1</sup> resulted in dramatic reductions in acidity, sulphate, and dissolved-iron concentrations of discharge water for 3-6 months (Monticello and Finnerty, 1985).

Application of ProMac, a bactericide that can be applied both as a spray and in controlled-release monolithic pellets has been successful in controlling *A. ferrooxidans* by destroying its protective outer coating, making it susceptible to the acid it produces (Sanda, 1989). Controlled release bactericides including ProMac inhibited *A. ferrooxidans* and promoted regeneration of a mining site (Sobek, 1987; Sobek *et al.*, 1990).

## B. Biological Control

Inundation of pyritic material and acid sulphate soils or of disused mine shafts has been suggested (Backes *et al.*, 1986; Evangelou and Zhang, 1995), since no significant growth of *A. ferrooxidans* has been demonstrated to occur in water-saturated environments (Kleinman and Crerar, 1979). A similar concept has been applied to the construction of wetlands to receive AMD in an anaerobic environment (Evangelou and Zhang, 1995). This environment encourages the activity of sulphate-reducing bacteria and so reduces acidity. Most of the hydrogen sulphide produced by these bacteria react with heavy metals to yield insoluble precipitates. Typical wetlands however, may not have sufficient permeability to take full advantage of this process.

Canada's Natural Resources Department 'Centre for Mineral and Energy Technology' (CANMET) proposed to develop an anaerobic "sulphuretum" for mitigation of acidic mine drainage by envisioning construction of a system of drainage ditches to control the flow of effluent through a bed of straw (McCready, 1991). As aerobic degradation of the straw proceeds, the sugars released will be fermented to organic acids by acidophilic heterotrophs. The organic acids will then be utilised by anaerobic sulphate reducers to reduce the sulphate in the effluent to hydrogen sulphide. Hydrogen sulphide percolating through a water column will precipitate dissolved metal ions as metal sulphides. Microbially produced CO<sub>2</sub> assists in buffering the system and provides a carbon source that may be combined with excess hydrogen by the methanogens to produce methane. In a laboratory study, the pH of the incoming liquid was 3.5 and after passage through the treatment zone it rose to pH 8.1. This process achieved a 65% reduction in the sulphate concentration and metals were not detected in the effluent.

Christison *et al.* (1985) reported that an unidentified zooflagellate was an effective predator, reducing the population of *A. ferrooxidans* from 10<sup>8</sup> cells/ml to 10<sup>2</sup> cells/ml within 18 hours at pH 2.3. Rotifers, as well as flagellated and ciliated protozoa were recorded as significant predators of *A. ferrooxidans* (McCready, 1987) but were incapable of eradicating them in liquid culture; moreover their large size makes it difficult for them to pursue their prey in interstitial spaces of mine tailings.

Padival *et al.*, (1995) found that an unidentified strain of yeast introduced into continuously stirred tank reactors with *H. neapolitanus* or *A. thiooxidans* resulted in a 99% decrease in the population of the latter. The effect on these thiobacilli was enhanced by limitation of nitrogen. The results suggest that strategies based on the competitive displacement of thiobacilli to inhibit corrosion of concrete sewers may be feasible.

## 2. Description of Detection and Monitoring Techniques, Including Specificity, Sensitivity and Reliability

### A. Thallous sulphide test

The ability to oxidise thiosulphates to sulphates with the production of elemental sulphur can be utilised to distinguish the sulphur-producing bacteria including *Acidithiobacillus*, from natural samples (Galizzi and Ferrari, 1976). Thallous sulphide paper moistened with pyridine is pressed onto agar plates with the colonies to be tested and then placed in dilute (0.12 N) nitric acid. The black thallous sulphide paper is bleached except in the presence of free sulphur, due to the presence of thallous polysulphides. If

sulphur is present, a brown spot is left at the site of the replicated colony. This test specifically enabled quantification of *T. thioparus* and *A. thiooxidans* in natural samples.

### B. Molecular probes

There are some specific problems associated with the identification and quantitation of micro-organisms in biohydrometallurgical operations (Yates and Holmes, 1986). The numerous species of autotrophic and heterotrophic bacteria may be morphologically similar and are often difficult to purify since they grow poorly or not at all on solid media. Analysis may be frequently aggravated by the presence of small rock particles and by the production of ferric precipitates. Molecular probes using cloned DNA sequences in Southern Blots and Dot blots, could distinguish between *A. ferrooxidans* and other species (*Acidiphilium acidophilum*, *Starkeya novella* and *T. thioparus*) as well as recognising several strains of *A. ferrooxidans*. This technique could, moreover, detect as few as  $10^3$  bacterial cells of a given species. The reverse sample genome probing (RSGP) technique was used by Léveillé *et al.* (2001) to monitor the presence of *Acidithiobacillus* species in AMD environments. Another genomic tool such as fluorescent *in situ* hybridisation (FISH) was successfully used in the laboratory to detect strains of *A. ferrooxidans* in an ADM environment (Leduc, personal communication).

### C. Polymerase-chain-reaction (PCR) and related methods

Recently PCR has been used in the detection and identification of *Acidithiobacillus* and other sulphur bacteria. Strains of *A. ferrooxidans* were differentiated by use of RAPD (random primer amplified polymorphic DNA) (Novo *et al.* 1996). Extending this observation, Selenska-Pobell *et al.* (1998) used genomic fingerprinting in the form of RAPD, rep-APD (repetitive primer amplified polymorphic DNA) and ARDREA (amplified ribosomal DNA restriction enzyme analysis) to distinguish four strains of *A. ferrooxidans*, and one strain each of *A. thiooxidans*, *E. coli*, *Burkholderia cepacia*, *T. thioparus* and *Thiomonas cuprinus*. The procedures not only discriminated between the different species but also suggested that one of the strains of *A. ferrooxidans* was only distantly related to the three others. This variable sequence homology was attributed by the authors to the greater ability of the variant strain to accumulate uranium, although all strains were isolated for some ability to do this. Such variability suggests that more than one method should be used to identify or distinguish different strains or species of thiobacilli.

The differentiation of one strain from the others tested may not be surprising since the strains were isolated from different strata and Novo *et al.* (1996) suggested that strains of *A. ferrooxidans* isolated from different micro-environmental sources could give varying patterns on RAPD.

### D. Isolation Media

Most sulphur bacteria can be isolated from natural habitats by the use of mineral media containing elemental sulphur or thiosulphate as an energy substrate. Use of media of different pH will assist differential selection of the neutrophilic and acidophilic species, whereas use of acid ferrous sulphate medium will frequently select for *A. ferrooxidans* (Kelly and Harrison, 1989).

A procedure has been described for the enrichment of facultatively autotrophic, mixotrophic bacteria, using a continuous flow chemostat provided with both organic and inorganic substrates (Gottschal and Kuenen, 1980). This provides a means of avoiding the predomination of heterotrophs in standard batch enrichment media containing supplements such as thiosulfate and glucose or acetate. In the latter, a mixture of obligatory chemolithotrophic thiobacilli and chemoorganotrophs normally develops. Harrison (1984) has described a general medium for cultivation of acidophilic bacteria comprising a basal mineral salts solution (MS) with the following (%w/v):

Media composition (per litre)

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.200
KCl	0.010
K <sub>3</sub> HPO <sub>4</sub>	0.025
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.025
Ca(NO <sub>3</sub> ) <sub>2</sub>	0.001

The pH is adjusted to pH 2-4 with 1N H<sub>2</sub>SO<sub>4</sub>.

Johnson (1995a) has since reviewed various solid media formulations for acidophilic bacteria published in the literature. The use of agarose, a purified derivative of agar, was recommended to overcome the fastidiousness of thiobacilli in this and other media, as well as the use of a double-layered medium, the underlayer incorporating an acidophilic heterotroph. The latter significantly lowered the proportion of monosaccharides and resulted in a dramatic increase in plating efficiency of most strains of *A. ferrooxidans*.

The basic medium recommended comprised ferrous sulphate and tryptone soya broth and potassium tetrathionate, and enabled differentiation and identification of isolates of several iron-oxidising bacteria based on colony characteristics.

*Acidithiobacillus albertensis*

This species can be grown on the media suitable for *A. thiooxidans* (Kuenen *et al.*, 1992) (see below). The pH should not be lower than pH 2.0.

*Acidithiobacillus caldus*

Hallberg and Lindstrom (1994) used tetrathionate as a main growth substrate adjusted to pH 2 and held at 32 °C or 52 °C according to the strain.

*Acidithiobacillus ferrooxidans*

This species does not readily form colonies on standard agar media because it is inhibited by some of the organic compounds found in unpurified agar (Holmes and Yates, 1990). The ability to form colonies on a solid medium is a necessary precondition for growth studies and for strain development. This problem has been overcome by substituting pure agarose for the routine agar medium or by growing the bacterium on membrane filters placed on the solid agar medium (Tuovinen and Kelly, 1973).

Growth of this species on solid media can be enhanced by the addition of small quantities of a surfactant such as Tween 80 (Garcia *et al.*, 1992). Use of ferrous sulphate (FeSO<sub>4</sub>·7H<sub>2</sub>O) with other basal salts has also been successful in enhancing the growth of the bacterium (Kuenen *et al.*, 1992; Harrison, 1984; Visca *et al.*, 1989). One of the *Thiobacillus* solid media, TSM-1, developed by Visca *et al.*, (1989), produced discrete and easily countable colonies and could be used for the isolation of single clones.

The ferrous sulphate medium is given by Kuenen *et al.* (1992) as follows:

Solution I (per litre)

K <sub>2</sub> HPO <sub>4</sub>	0.5 g.
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.5 g
H <sub>2</sub> SO <sub>4</sub>	5 ml of a 15N solution

Solution II (per litre)

FeSO <sub>4</sub> .7H <sub>2</sub> O	0.5 g
H <sub>2</sub> SO <sub>4</sub>	150 ml of a 15N solution

Four parts of solution I are mixed with 1 part of solution II to give a medium containing 120 mMFe<sup>2+</sup>. Formation of iron precipitates can be avoided by lowering the pH through successive subcultures to a value of 1.3 with H<sub>2</sub>SO<sub>4</sub>.

**Solid 2:2 medium for genetic manipulation of *A. ferrooxidans***

This medium was developed by Peng *et al.* (1994a) for the isolation of mutants. The medium, prepared in four parts, contains ferrous sulphate and sodium thiosulphate as energy sources for the growth of the bacterium at pH 4.6–4.8. Strains resistant to kanamycin and streptomycin could be obtained by incorporating increasing concentrations of these antibiotics in the medium and selecting out those colonies which developed. The medium encouraged the growth of a wide morphological range of colonies. It is also possible to introduce plasmids into bacterial cells using this medium and to develop strains resistant to heavy metals.

*Acidithiobacillus thiooxidans*

Kuenen *et al.* (1992) list the isolation and growth media for this species as follows:

Media (per Litre)

K <sub>2</sub> HPO <sub>4</sub>	3.5 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.3 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.5 g
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.018 g
CaCl	0.25 g
Flowers of Sulphur	5.0 g
Adjust to a pH of 4.5	

Both *A. ferrooxidans* and *A. thiooxidans* may be cultivated in minimal salts (MS) containing 1% powdered sulphur. Sulphur melts at ~113 °C, so the MS-sulphur slurry is sterilised by heating at 105 °C for one half-hour on two successive days (Harrison, 1984). These acidophilic thiobacilli have also been isolated on a mineral basal salts medium supplemented with ferrous sulphate and substituting 0.4% Gelrite, a bacterial polysaccharide, for agar (Khalid *et al.*, 1993). Dark brown circular colonies have been observed to develop on this medium within 72–96 hours.

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<i>Capsicum annuum</i> Complex	Korea	2006	ENV/JM/MONO(2006)2
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